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Membrane filtration procedures for the quantification of Candida albicans and Giardia lamblia from water

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MEMBRANE FILTRATION PROCEDURES FOR THE QUANTIFICATION OF *CANDIDA ALBICANS* AND *GIARDIA LAMBLIA* FROM WATER

by

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Membrane Filtration Procedures for the Quantification of *Candida albicans* and *Giardia lamblia* from Water (82 pp.)

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The microbial hazard assessment of water quality involves the extrapolation from an organism found in the polluted environment to the associated disease. Coliforms were adopted as an indicator of fecal contamination in the late 1800s but it is frequently difficult to relate the coliform number recovered to the associated health risk, as with giardiasis. *Candida albicans* has been isolated from water and has potential as an indicator of water quality but little quantitative data has been collected due to the lack of a adequate medium for the enumeration of this organism. In this report, the membrane filtration procedures are described for the quantification of *C. albicans* and *Giardia lamblia* from untreated surface water.

The selection and differentiation of *C. albicans* in pure culture and in bifloral cultures with *C. tropicalis, C. parapsilosis, Saccharomyces cerevisiae, Torulopsis candida* and a *Cryptococcus* sp. was assessed using four experimental membrane filtration media containing one of four indicators; bismuth-sulfite, molybdic acid, tetrazolium chloride or nitro-blue tetrazolium. Recovery on the experimental media was compared to that achieved on a non-inhibitory glucose-peptone medium. The nitro-blue tetrazolium medium yielded an average *C. albicans* recovery rate of 100% and background organisms found in natural waters were reduced by at least three orders of magnitude. Excellent selectivity was confirmed by filtering surface water seeded with *C. albicans*; 98% of typical *C. albicans* colonies were verified as such using carbohydrate assimilation patterns and 0% of the atypical colonies selected for identification were verified as *C. albicans*.

The filtration of water seeded with *G. lamblia* cysts followed by zinc sulfate concentration yielded a 96% recovery rate. No correlation between the occurrence and density of *G. lamblia* and *C. albicans* could be formulated as no indigenous *G. lamblia* cysts were detected. However, the isolation of *Capillaria* and *Toxocara* eggs indicated that the membrane filtration and zinc sulfate concentration procedures developed were sound.

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ABBREVIATIONS

BSM	bismuth-sulfite medium, membrane filtration
CFU	colony forming unit
D^2	dispersion index
MF	membrane filtration
NBT	nitro-blue tetrazolium medium, membrane filtration
PMA	phosphomolybdic acid medium, membrane filtration
PBS	phosphate buffered saline
P	probability
SGA	Sabouraud glucose agar
TTC	tri-phenyl tetrazolium chloride medium, membrane filtration

I. INTRODUCTION

Historical Background

The microbial hazard assessment of water quality began with the work of von Fritsch and Escherich in the late 1800s when the presence of coliforms in water was recognized as being indicative of fecal contamination (40). Water is generally accepted as the most prevalent disease-carrying agent known, and the most common disease sympton is gastroenteritis. Reviews of waterborne disease outbreaks in the United Ststes for the periods of 1920 to 1936 (59), and 1938 to 1946 (45), revealed that gastroenteritis was by far the most frequently reported waterborne related illness, eighty-eight and ninety-one percent respectively; yet the etiological agent is rarely identified. Attempts to isolate the pathogen from a suspected water source are frequently unsuccessful.

The numbers of enteric pathogens present in a fecal specimen are comparatively small when considering the great numbers of normal excremental organisms present, such as fecal coliforms (12,108). The detection of coliforms may be successful when some pathogens such as *Salmonella*, *Shigella*, enteric viruses and enteric cysts cannot be detected due to the low number of pathogens present, a low pathogen survival index in the aqueous

environment, or difficulty in recovering the pathogen from the aqueous environment.

The introduction of the multiple tube procedure in 1892 allowed for the enumeration of coliforms in water, but depended upon a large number of samples to insure result accuracy. Coliform testing sensitivity was greatly enhanced in the late 1930s by increasing possible sample size with the use of the membrane filter (53,57,135). By 1962, membrane filtration had been accepted as the standard method for the detection and the enumeration of coliforms. Although membrane filtration greatly facilitated the quantification of coliforms in water, the relationship between the coliform number recovered and the associated health risk remained somewhat of an enigma.

The recovery of stressed coliforms on membrane filters is often erratic; numbers may vary as mush as forty-four to ninety-two percent of coliform number determined by plate count procedures on identical samples (25,40,62). The discrepancies in results have been attributed to cell damage and to the inhibition of cell growth by the pore structure inherent to the membrane filter (120,125). The coliform number recovered immediately prior to or during a waterborne disease outbreak may indicate that no health risk exists (34,63,111,118). The enrichment of a stressed culture in a non-selective liquid medium prior to filtration has been used successfully to improve coliform recovery (9,25,87,121), but the results may continue to be

erratic or consistently lower than the actual number present as determined by the plate count procedure (13,40,71). Also, a high total bacterial count increases the competition for growth on the membrane surface and may significantly alter the results by decreasing the coliform number recovered (23,55,114), or by invalidating the coliform identification procedures by producing a high frequency of false positive results (11,108). Finally, competition for survival in the aqueous environment and specific environmental conditions may combine to eliminate possible correlation of numbers of coliforms recovered with numbers of any specific pathogen present. The relationship between coliforms and enteric pathogens is inconsistently effected by the length of exposure to water (50,98), the temperature of the water (68,106), the total bacterial count (51,117), and the water disinfection procedures (76,84,114).

The relationship between the yeasts present in water and the coliform number is not well understood, but results from analyses of ten thousand river water samples indicated a positive correlation between the number of yeasts present and the extent of organic pollution (101,105,115). The yeasts present are dominated by the genera *Candida*, *Rhodotorula* and *Debaryomyces* and there is an express need to establish a reliable correlation between the presence of specific fungal populations in water and the degree of pollution.

The genus *Candida* was recognized to be a mucosal

saprophyte by Langenbeck in 1938 (26). The most frequent human isolate is the species, *C. albicans* (*Oidium albicans* Robins, 1853; *C. albicans* Berkhout, 1923), which occurs in eighty-five percent of human alimentary canals (2,26,85, 93). The presence of *C. albicans* in water generally poses no health risk, in itself, as it is an opportunistic pathogen; however, its presence in water is a specific indication of organic pollution of animal origin (3,28,48,124).

The isolation of *C. albicans* from soil (39,82,127), and from vegetation (130), indicate that the yeast can survive for an extended period of time outside the animal host. It survives in distilled water for six to eight weeks (42,44), and for longer periods of time in water with an increased organic content (5,23,28,48,112).

The efficiency of recovery of yeasts from water using the membrane filter is equal or superior to that of the plate count procedure (19,109,133). This is the reverse of the situation with coliform bacteria and is believed to result from the more rigid structure of the yeast cell wall and its larger cell size.

The high incidence of *C. albicans*, its ability to survive for long periods of time outside the animal host, and the high recovery rate of yeasts on the membrane filter may make the quantification of *C. albicans* from water useful in the assessment of water quality in instances where coliform number recovered bears no simple, nor predictable, relationship with health risk.

An enteric pathogen difficult to isolate from the aqueous environment is *Giardia lamblia*. This organism poses a continual health problem and is the parasite most frequently reported in public health labs in the United States (72,73). The prevalence of *Giardia* in the United States populace ranges from one to twenty percent, depending on the socioeconomic status of the area (10,31,38, 52,97,102). Fifty percent of giardiasis outbreaks result from the ingestion of untreated ground or surface water (34,137), but also from ingestion of water receiving only minimal disinfection, or water subject to faulty sewage disposal or reclamation (32,80).

The first confirmed giardiasis outbreak reported in the United States occurred in Aspen, Colorado during 1965-66, and was attributed to the leakage of sewage into water wells (89). Considering that the prevalence in the local population reached five percent (102), and that carriers may excrete five hundred to two thousand cysts per gram of feces (37,92), the successful isolation of *G. lamblia* cysts from raw sewage was undoubtedly enhanced by the large numbers of cysts present.

Although thousands of persons may become ill during a giardiasis outbreak, cysts are rarely isolated from the suspected potable water source due to dilution of contamination by the time sampling is started (132). Also, the filter systems designed to increase cyst recovery through large volume sampling have exhibited sampling losses of up

to forty-eight percent due to retention of cysts in the filter fibers (74,75).

Several studies have shown that negative results on coliform testing do not insure that the water poses no giardiasis hazard. Coliform counts performed during giardiasis outbreaks in Utah (65), Colorado (7), and New Hampshire (91), were either below risk levels or demonstrated that the water was consistently coliform free. Because the procedures for isolation of *Giardia* cysts from water are often unsuccessful, the risk of giardiasis should be correlated with an indicator other than coliforms; quantitative *C. albicans* data should be considered for use in assessing the health risk of giardiasis from potable or free waters.

The presence of *C. albicans* or *G. lamblia* in water is specific evidence of contamination from human or animal excrement (3,124). Both organisms exhibit long-term survival outside the animal host (3,39,61,100,112,136), and both are host inspecific (38,76,99,127), with host organisms sharing the same habitats.

The use of *C. albicans* as an indicator of potential giardiasis may be successful where correlation with coliforms has failed. Following inoculation of water, a coliform population may exhibit a rapid decline to zero within days (40,98), or may proliferate to 650% of the inoculum density (40,68,126). On the other hand, *C. albicans* demonstrates only restricted budding in the aqueous environment (60,114),

and tends to survive well. Therefore, the number of *C. albicans* cells recovered may provide a more accurate representation of the extent of fecal contamination, and the associated health risk, than does the number of coliforms recovered.

Statement of Thesis

Terrestrial run-off is the major factor in determining the character of the microbiological population in water (4,17,23,48,112,126). The Rattlesnake Creek drainage and the Missoula irrigation ditch systems are two water sources that are significantly affected by terrestrial run-off. As water enters the Missoula valley and flows through cultivated, grazed or domiciled areas, the wastes from human and animal activities are shed directly or washed into the water by rain, snowmelt or local irrigation practices. The expanse of surface water of variable quality proximal to Missoula provides an excellent resource for the examination and the evaluation of membrane filtration procedures for the quantification of *C. albicans* and *G. lamblia* from water.

Membrane filtration has been used extensively to isolate yeasts from the aqueous environment (1,27,36,96,116,128,129), but quantitative data is meager (15,16,17,19,112,115), due to the lack of an adequate medium for the enumeration of yeasts from water. As *C. albicans* is a medically important yeast, several media have been developed for the isolation of this

organism from clinical specimens, but these media either fail to prevent the rapid overgrowth of bacteria and/or filamentous fungi found in natural waters (19,64), or are low in nutritive value so as to prompt chlamydospore formation (21,91). Media low in nutritive value are not conducive to a high rate of recovery of stressed organisms from the aqueous environment.

In this project, four experimental membrane filtration media were evaluated as to their value in the isolation and enumeration of *C. albicans* from water. Three of the four media are modifications of solid plating media developed for the isolation of *Candida* species from human sources and contain bismuth-sulfite, molybdic acid, or tetrazolium chloride as growth indicators. The fourth experimental medium consists of a fourth, and novel, indicator, nitro-blue tetrazolium, added to a modified basal medium. Various test modifications of the media include omission of agar, doubling the concentration of all nutritional constituents (25), and the addition of antibiotics to control bacterial and filamentous fungal growth. The experimental membrane filtration medium found to be the most selective and sensitive was used to quantify *C. albicans* populations from selected surface water sources.

Also examined in this project were membrane filtration and zinc sulfate concentration procedures for the detection of *G. lamblia* cysts in water. Such methodologies may provide a means for the quantification of cysts that is

more sensitive than is the culturing of cysts (24,58). Filtration and concentration procedures were effective in confirming the presence of *Giardia* in potable water during a giardiasis epidemic in Rome, New York (69,78, 113); in the same circumstance, microscopic examination of sediment filtered from over one million liters of water was not successful. In this research, the membrane filtration and zinc sulfate concentration procedures were used to examine several potentially polluted surface water sources for the presence of *G. lamblia* cysts, and the accuracy of quantification was determined.

Water sources which yield *G. lamblia* cysts were then processed for the quantification of *C. albicans* and correlation between the occurrence and density of these two organisms was examined.

II. MATERIALS AND METHODS

Membrane Filtration Equipment

The membrane filter apparatus used throughout was a 500-ml, single filter, vacuum style funnel (Millipore Filter Corp). The membrane filters were GN-6 Metricel, 0.45 micrometers, white with grids (Gelman Instrument Co) and are recommended for high yeast recovery (109,120). The dishware used were 50 x 9 mm plastic petri dishes with tight fitting lids (Falcon).

Yeast Membrane Filtration

Yeast cultures. Strains of the yeasts *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *Saccharomyces cerevisiae*, *Torulopsis candida* and a *Cryptococcus* sp. were obtained from the culture collection of Dr. John Taylor, University of Montana. The yeasts were maintained on Sabouraud glucose agar (Difco) slants at 25°C and were transferred at bi-weekly intervals.

Yeast cell suspensions. Yeast cell suspensions were prepared from 48-hour cultures grown at 25°C on Sabouraud glucose agar (SGA) plates. Cell suspensions were made by flooding the plates with 5.0 ml phosphate buffered saline (PBS), pH 7.2, and agitating the agar surface with a bent

glass rod. The cell suspension was transferred to a glass vial and was vortexed for a minimum of two minutes to minimize cell clumping.

The optical density of each yeast cell suspension was adjusted to 0.15 at 600 nm (Coleman Junior II Spectrophotometer) and serial dilutions were plated in triplicate on SGA plates and incubated at 25°C for 72 hours. The dilution with the appropriate number of yeast cells per milliliter, that suspension with a density easily adjusted to 50 to 200 cells per ml, was selected for each yeast species and was stored at 6°C for no longer than one week.

Membrane filtration procedure. Aliquots of the cold-stressed yeast cell suspensions were added to 20 ml PBS in the assembled filter apparatus. This volume was filtered and the sides of the funnel were rinsed twice with an additional 20 ml PBS.

The membrane was removed from the filter apparatus with sterile forceps and placed upon a filter pad saturated with 1.8 ml of medium (88) cooled to 10-15°C to avoid temperature stress of the starved yeast cells in suspension (83). The petri dish lids were affixed firmly to maintain a high relative humidity for the improved recovery of *C. albicans* (93).

Assessment of yeast recovery on the membrane filter. The effect of the membrane filter itself on the recovery of *C. albicans* was assessed by cultivating triplicate filtered

aliquots on a non-selective glucose-peptone yeast broth (see Appendix). One hundred percent recovery was assessed by spread-plating parallel aliquots on SGA plates. All cell suspensions were vortexed briefly prior to any enumeration procedure to minimize cell clumping and all filters and plates were incubated at 25°C for 48 hours.

Experimental Membrane Filtration Media

Bismuth-sulfite medium. One hundred ml of Bacto BiGGy agar was prepared double strength and centrifuged at 3,000 rpm for 20 minutes to sediment out the agar. Fifty ml of the supernatant was withdrawn with a pipette, 3 g of glucose was added, and the medium was heated to the boiling point. The medium was stored in the dark at 6°C and was used within 72 hours.

Phosphomolybdic acid medium. The basal medium consisted of 3% proteose peptone (Difco), 0.2% yeast extract (Difco), and 8% sucrose (Difco). After adjusting the pH to 7.6 with 8% aqueous NaOH, the medium was autoclaved for 15 minutes at 15 pounds pressure. To 50 ml of the basal medium, cooled to 50 to 55°C, was added 1.5 ml of a 12% solution of filter-sterilized phosphomolybdic acid (J.T. Baker Chemical Co). This medium was stored in the dark, at 6°C and was used within 72 hours. Prior to saturating the filter pad, the medium was agitated to suspend the precipitate. An even distribution of the

precipitate over the pad surface was obvious due to uniform coloration.

Tetrazolium chloride medium. The basal medium consisted of 2% neopeptone (Difco), 0.2% yeast extract, and 8% glucose (Becton, Dickinson and Co). After adjusting the pH to 6.0 with 8% aqueous NaOH, the medium was autoclaved for 15 minutes at 15 pounds pressure. To 50 ml of the basal medium, cooled to 50 to 55°C, was added 1.0 ml of a 2.5% solution of filter-sterilized 2,3,5-triphenyl-2H-tetrazolium chloride (J.T. Baker Chemical Co). This medium was stored in the dark, at 6°C and was used within 72 hours.

Nitro-blue tetrazolium medium. The basal medium consisted of 2% phytone peptone (Difco), 0.2% yeast extract, and 8% glucose. After adjusting the pH to 6.0 with 8% aqueous NaOH, the medium was autoclaved for 15 minutes at 15 pounds pressure. To 50 ml of the basal medium, cooled to 50 to 55°C, was added 1.0 ml of a 2.5% solution of filter-sterilized tetrazolium blue (Nutritional Chemical Corp). This medium was stored in the dark, at 6°C and was used within 72 hours.

Incubation temperature. The yeast cultures cultivated on either the bismuth-sulfite or phosphomolybdic acid media were incubated at 36°C (95,103), and the yeast cultures cultivated on either the tetrazolium chloride or nitro-blue tetrazolium media were incubated at 25°C. All

yeasts cultivated on a non-selective media were incubated at 25°C.

Antibiotics. The experimental membrane filtration media were sometimes further modified with the addition of antibiotics. Cycloheximide at 0.4 mg/ml (6,56) and chloramphenicol at 0.05 mg/ml (8,21) have been demonstrated to be non-inhibitory to *C. albicans* while selecting against other yeasts and bacteria. The effect of the antibiotics was assessed by comparing the recovery of all six yeast species on the experimental membrane filtration media containing one or both antibiotics with yeast recovery on a non-selective glucose-peptone medium. Triplicate parallel aliquots were incubated for 48 hours at the appropriate temperature.

Assessment of Membrane/Media Interaction

The possibility of any media constituent reacting with the membrane filter to influence the recovery of *C. albicans* was examined. Equal aliquots of the yeast cell suspensions were either filtered and cultivated on the experimental membrane filtration media, or were spread-plated on the solid equivalent of the membrane filtration media (see Appendix).

Application of Yeast Enumeration Procedures

Collection of samples and sampling sites. Water

samples were collected at bi-weekly intervals along the Rattlesnake Creek drainage and the Missoula irrigation ditch system by below the surface grab sampling using 500-ml and 2000-ml glass flasks. The samples were transported from the collection sites (Figure 1 and Figure 2) to the lab within one hour of collection and were either processed immediately or were held at 6°C for no longer than 4 hours.

Rapid demonstration of *C. albicans*. Each water sample was processed for the rapid demonstration of *C. albicans*. A 100-ml portion of each sample was filtered and the membrane was transferred to a small culture dish and was inverted into 2 ml of Mycosel broth (Becton, Dickinson and Co) and incubated at 36°C. Aliquots of the agitated broth culture were removed after 24 hours and were streaked for isolation on duplicate BiGGy agar plates. The plates were examined after 48 hours incubation at 36°C and colonies which resembled *C. albicans* were picked for identification by carbohydrate assimilation patterns using the Analytab Products 20-C yeasts system (Plainview, NY). Preliminary trials using serial dilutions of *C. albicans* in PBS indicated that the presence of four *C. albicans* cells could be detected within 72 hours.

Inhibition of background organisms. The effect of background organisms on the recovery of *C. albicans* was assessed by seeding equal volumes of PBS and pooled surface

Figure 1. Sampling sites along the Rattlesnake Creek drainage and the Missoula irrigation ditch system.

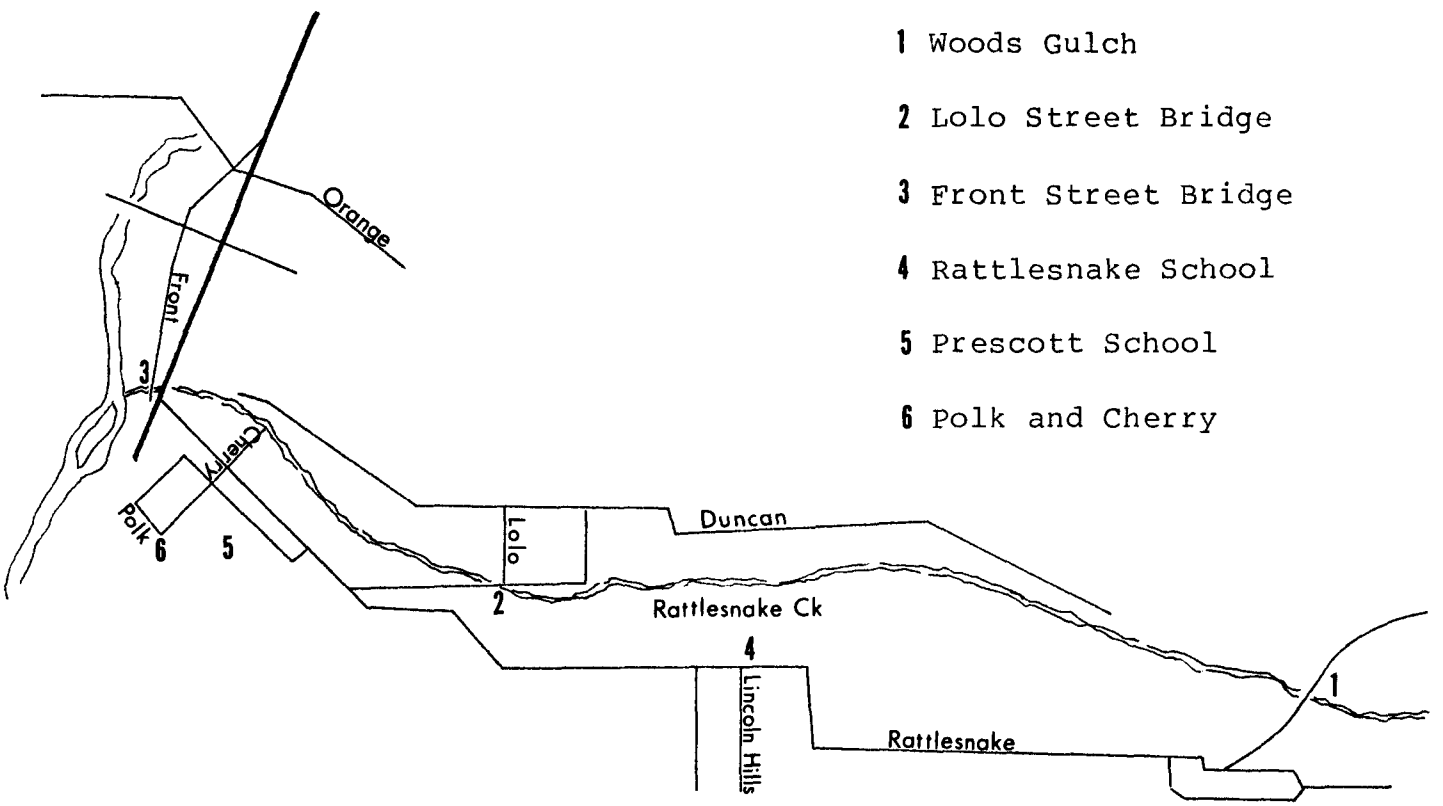
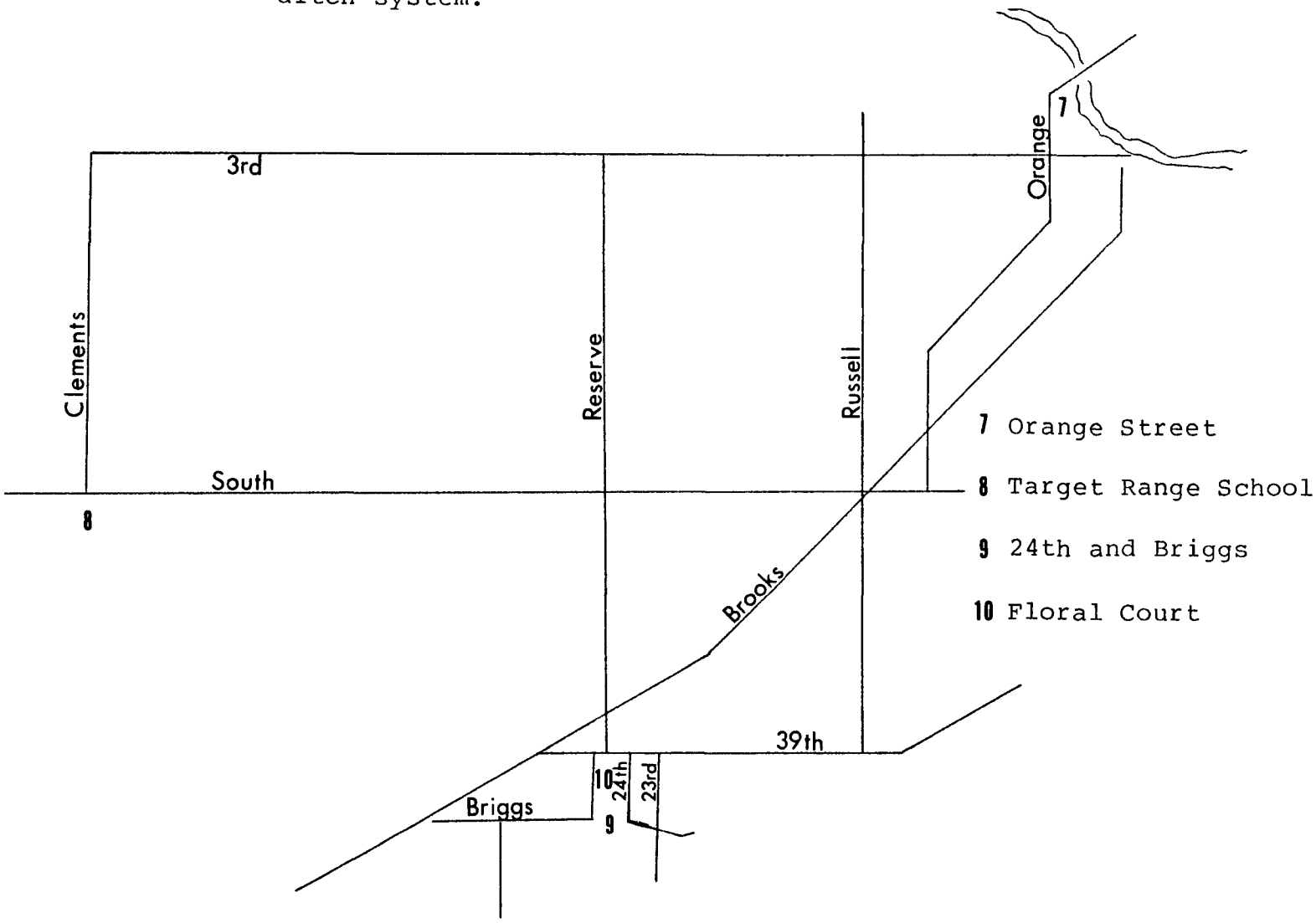


Figure 2. Additional sampling sites along the Missoula irrigation ditch system.



water from three sources with equal numbers of *C. albicans* cells. Triplicate 100-ml aliquots of the seeded samples were filtered and the membrane filters cultivated on membrane filtration media containing antibiotics. The colonies which demonstrated typical *C. albicans* characteristics after 48 hours incubation were streaked for isolation on BiGGy agar plates, incubated for 48 hours at 36°C and a few selected colonies were identified by carbohydrate assimilation patterns.

Enumeration of indigenous *C. albicans*. Sample filtration volumes were selected to yield an optimum 80 to 200 colonies per membrane (54). During a period of peak density, samples were collected at weekly intervals and 500-ml samples were filtered and cultivated on the nitro-blue tetrazolium medium for the quantification of indigenous *C. albicans*.

Assessment of Cyst Recovery from the Membrane Surface

The pilot analyses to determine the recovery of *G. lamblia* cysts from the membrane filter surface were done with *S. cerevisiae* cells to approximate the size and shape of cysts.

The yeast was grown at room temperature on a rotary shaker in 50 ml of Sabouraud glucose broth (Difco) and the cells were harvested after 48 hours by centrifugation at 2,000 rpm for 5 minutes and decanting the supernatant. The

cells were resuspended in cold PBS, pH 7.2, and were vortexed for 2 minutes to minimize cell clumping. The yeast cell density was determined using a hemacytometer and was adjusted to approximately 200 x 10^5 cell/ml with additional PBS. A budding blastospore was consistently counted as a single cell.

An aliquot of the yeast cell suspension of known density was added to a minimum of 20 ml PBS in the assembled filter apparatus. This volume was filtered and the sides of the funnel were rinsed for 4 seconds with a continuous stream of PBS. The membrane was then removed to the bottom of a petri dish and was flooded with a know small volume of PBS. The dish was tilted and the cells were washed from the membrane by repeatedly flushing the surface with the cell suspension using a Pasteur pipette with bulb.

The recovery of *S. cerevisiae* cells was determined for three procedure trials, each run in triplicate, using variable volumes of PBS to resuspend the yeast cells. The densities of the resulting suspensions were determined using a hemacytometer.

Trial 1: A 0.5-ml aliquot was filtered, the membrane was flooded a single time with 1.0 ml PBS, and the cell suspension was retained in the petri dish.

Trial 2: A 2.0-ml aliquot was filtered, the membrane was flooded a single time with 5.0 ml PBS, and the cell suspension was retained in the petri dish.

Trial 3: A 2.0-ml aliquot was filtered, the membrane was flooded three times with 1.0 ml PBS, and each resulting cell suspension was pooled in an empty petri dish.

Cyst Enumeration Procedures

Cyst suspension preparation. A fecal suspension containing formalin-preserved *G. lamblia* cysts was obtained from the Western Montana Clinic. A quantity of fecal debris was removed by centrifugation at 2,500 rpm for one minute and decanting the supernatant. The pellet was resuspended in 10 ml phosphate buffer, pH 7.2, and centrifugation was repeated until the supernatant was clear.

Membrane filtration. A 0.5-ml aliquot of the fecal suspension was added to 20 ml of phosphate buffer in the assembled filter apparatus. This volume was filtered and the sides of the funnel were rinsed twice with 20 ml of phosphate buffer. The filter was removed to the bottom of a petri dish and was flooded with 1.0 ml of phosphate buffer. The cysts and the fecal debris were washed from the membrane surface as previously described for *S. cerevisiae* cells. The membrane was flooded a total of three times with 1.0 ml of phosphate buffer and the suspensions were pooled in a 12-ml conical centrifuge tube. The suspended material was reduced to a pellet by centrifugation at 2,500 rpm for 1 minute and decanting the supernatant.

Zinc sulfate concentration. The recovery of cysts from the membrane filter surface was assessed using zinc sulfate concentration to determine the cyst density of the fecal suspension prior to and following membrane filtration. The suspended material in the unfiltered fecal aliquot was reduced to a pellet by centrifugation at 2,500 rpm for 1 minute and decanting the supernatant.

The pellets recovered from filtered and unfiltered fecal aliquots were resuspended in 4 ml of zinc sulfate solution with a specific gravity of 1.20 (Scientific Inc., 1.000 - 1.400 hydrometer). The tube was then filled to the brim with additional zinc sulfate solution and a coverglass was added so that the underside touched the meniscus and no air bubbles were present. The tube was centrifuged at 2,500 rpm for 1 minute and the coverglass was removed with an upward motion and placed onto a slide into a drop of Lugol's iodine (see Appendix). A slide was examined microscopically immediately after preparation and the cysts were identified by their distinct morphological characteristics and counted.

Pre-filtration. A portion of the fecal suspension was pre-filtered to determine the effect of removing large debris on the recovery of cysts. A 5.0-ml aliquot was filtered through a 47 micrometer polypropylene filter (Gelman Sciences, Inc) moistened with phosphate buffer. The debris was resuspended by flooding the filter with

10 ml phosphate buffer and agitating the filter surface with a moistened artists brush. This volume was filtered and the debris was resuspended in 10 ml phosphate buffer four additional times. The pre-filtered fecal suspension was collected in a glass vial.

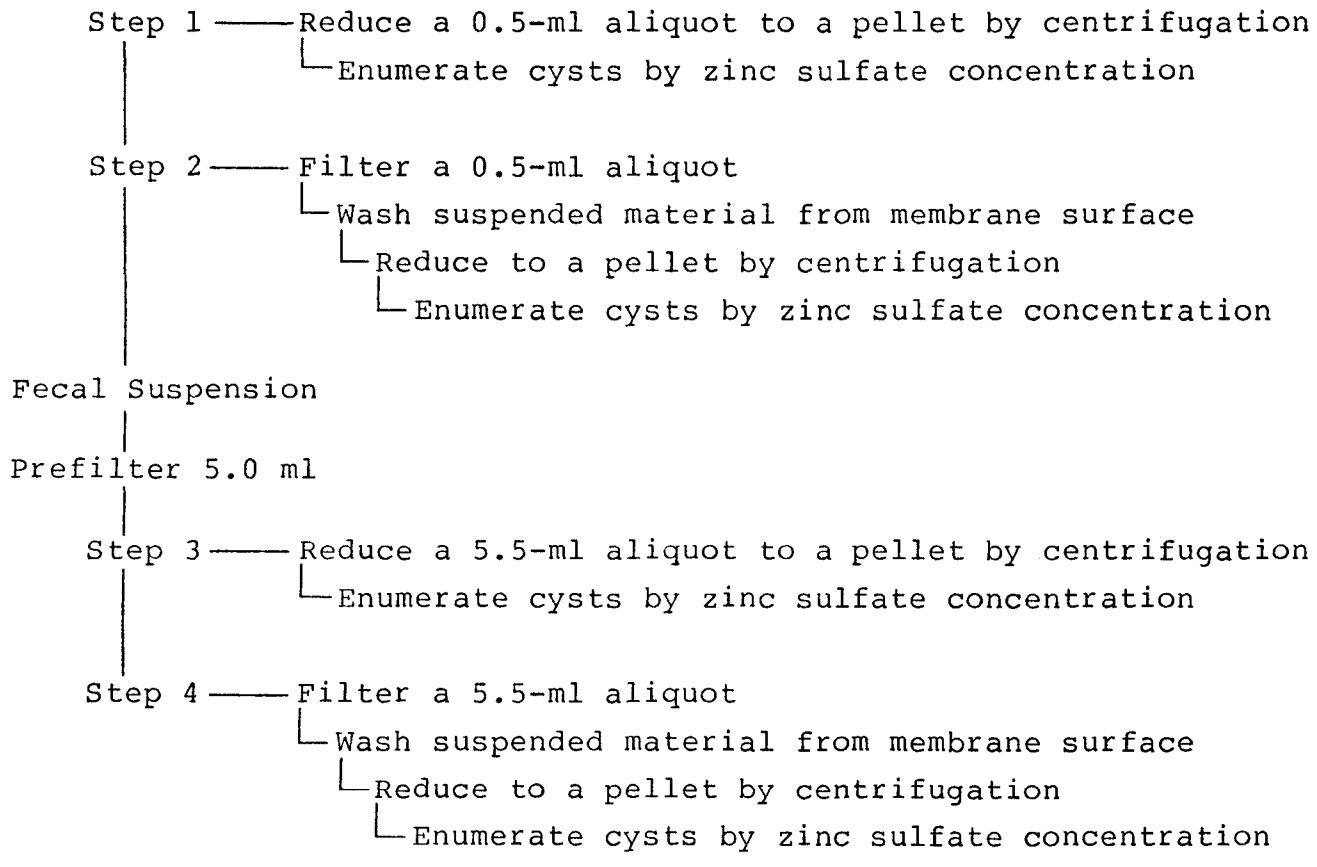
Maximum cyst recovery was assessed by reducing triplicate 5.5-ml aliquots to pellets by centrifugation and enumerating the cysts present using zinc sulfate concentration. The recovery of cysts from the membrane filter surface was assessed by filtering triplicate 5.5-ml aliquots, washing the suspended material from the membrane surface, pooling the resulting suspensions, and enumerating the cysts using zinc sulfate concentration.

The cyst enumeration procedures are outlined in Figure 3.

Application of Cyst Enumeration Procedures

Recovery of cysts from surface water. The recovery of *G. lamblia* cysts from surface water was assessed by seeding cyst fee ditch water with an aliquot of the fecal suspension for which cyst density had been determined using zinc sulfate concentration. Two 1,000-ml ditch water samples were seeded with approximately 600 and 1200 cysts. The volume of water to be filtered through a single membrane was dependent on water turbidity. If the filtration of 20 ml of the seeded water required more than 15 minutes, a

Figure 3. Procedures for the enumeration of *Giardia lamblia* cysts from prefiltered and non-prefiltered fecal suspensions.



new membrane was used to filter the remaining sample portion. The membrane filter washings from each filter were pooled into two centrifuge tubes and cyst recovery was assessed using zinc sulfate concentration.

Isolation of indigenous *G. lamblia* cysts. Surface water samples were collected using 2,000 ml glass flasks by below the surface grab sampling at four sites; Rattlesnake Creek at Lolo Street Bridge, Lafray Creek just prior to emptying into the Blackfoot River, Rock Creek, 10 miles from Interstate 90, and Wallace Pond, 4 miles east of Clinton. The water samples were transported from the collection sites to the lab within one hour of collection and were either processed immediately or were held at $6^{o}C$ for no longer than 12 hours.

The membrane filtration and zinc sulfate concentration procedures are the same as those described previously with one exception: the specific gravity of the zinc sulfate concentration solution was adjusted to 1.8 for the concentration of fresh cysts (see Appendix).

III. RESULTS

Recovery of *C. albicans* on the Membrane Filter

Effect of vortexing the yeast cell suspension. The suspensions of *C. albicans* cells which were vortexed for a 2-minute period demonstrated a minimal 200% increase in colony count (Table 1). As additional treatment did not alter the results, the initial vortexing period was deemed sufficient in eliminating the effect of cell dissolution on increased colony count.

Effect of the membrane filter on *C. albicans* recovery. The membrane filter itself had no deleterious effect on the recovery of *C. albicans*. Yeast cell recovery on the membrane filter using a non-inhibitory glucose-peptone medium was equal to or superior to recovery when the yeast was spread-plated on the SGA agar surface (Table 2).

Membrane/media interaction. The possibility of a constituent in the media reacting with membrane filter to effect the recovery of *C. albicans* was also eliminated. The recovery of *C. albicans* on the filter was equal to recovery when the yeast was spread-plated on equivalent solid plating media (Table 3). The selective properties exhibited by any of the four membrane filtration media are therefore properties of the media themselves and do not represent a membrane/media interaction.

Table 1. Effect of vortexing suspensions of *Candida albicans* cells prior to spread-plating and membrane filtration procedures to minimize cell clumping.

Volume of Cell Suspension Plated	Colony Count of Cell Suspension Not Vortexed				Colony Count of Cell Suspension Vortexed				Increase in Colony Count
	-1-	-2-	-3-	Mean	-1-	-2-	-3-	Mean	
0.2 ml	28	30	36	31	73	77	74	75	240%
0.4 ml	54	71	64	63	120	140	140	130	210%
0.8 ml	110	120	130	120	270	270	280	270	220%

Table 2. Comparison of recovery of *Candida albicans* when cells were spread-plated on the agar surface or cultivated on the membrane surface on a non-inhibitory glucose-peptone medium.

Volume of Cell Suspension Plated or Filtered	Colony Count from Spread-Plating				Colony Count from Membrane Filter				Recovery Comparison
	-1-	-2-	-3-	Mean	-1-	-2-	-3-	Mean	
0.5 ml	69	71	80	73	79	70	70	73	100%
1.0 ml	130	140	160	140	160	140	150	150	110%
1.5 ml	200	210	240	220	240	230	220	230	100%

Table 3. Comparison of recovery of *Candida albicans* using experimental membrane filtration media when cells were spread-plated on the agar surface or cultivated on the membrane surface.

	Colony Count Recovery from Spread-Plating			Colony Count Recovery from Membrane Filter			Recovery Comparison
	-1-	-2-	Mean	-1-	-2-	Mean	
Bismuth-Sulfite Medium, MF	200	180	190	200	180	190	100%
Phosphomolybdic Acid Medium, MF	170	180	180	190	180	180	100%
Tetrazolium Chloride Medium, MF	93	100	100	99	96	98	99%
Nitro-Blue Tetrazolium Medium, MF	120	120	120	130	120	120	100%

Differentiation on Membrane Filtration Media

Bismuth-sulfite medium. The differentiation of *Candida* species based on sulfite reduction has been well established (104,105). *Candida albicans* demonstrated the greatest degree of sulfite reduction producing dark or very dark brown, glossy, convex colonies which measured 3-5 mm in diameter (Table 4). In mixed culture, *C. albicans* could not be easily differentiated from either *C. tropicalis* or *C. parapsilosis*. The colony color which was characteristic of each of these three *Candida* species in pure culture was represented in the mixed cultures, but a significant number of colonies expressed an intermediate colony color variation and could not be identified with sufficient accuracy. When the incubation time was extended to 72 hours, it was noted that the *C. parapsilosis* colonies tended to flatten and were more readily differentiated from *C. albicans* which remained prominently raised.

Phosphomolybdic acid medium. On this medium, *C. albicans* colonies were dark navy blue, glossy, convex, and measured 1-3 mm in diameter. Molybdate is reduced in sequential steps from blue to green to brown products (30) and color variations among the six yeast species included navy blue, blue-green, blue-grey, green and tan (Table 5). These color differences remained distinct in mixed culture. Extending the incubation time to 72 hours diminished colony color differences; navy blue and blue-grey colonies became

Table 4. Differentiation among six yeast species in pure culture on the bismuth-sulfite medium, MF incubated at 36°C for 48 hours.

	Pigmentation	Diameter	Surface	Elevation
C. albicans	Very dark brown; solid color	3-5 mm	Glossy	Convex
C. tropicalis	Dark brown; solid color	3-5 mm	Glossy	Convex
C. parapsilosis	Medium brown; solid color	4-5 mm	Matte	Convex
S. cerevisiae	Tan; solid color	1 mm	Matte	Convex
T. candida	Light brown; solid color	2-3 mm	Glossy	Convex
Cryptococcus sp.	Medium brown; solid color	3-5 mm	Glossy	Convex

Table 5. Differentiation among six yeast species in pure culture on the phosphomolybdic acid medium, MF incubated at 36°C for 48 hours.

	Pigmentation	Diameter	Surface	Elevation
C. albicans	Navy blue or dark blue-green; solid color	1-3 mm	Glossy	Convex
C. tropicalis	Medium blue-green; darker in center	3-5 mm	Matte	Convex with flat edges
C. parapsilosis	Medium blue-grey; darker in center	1 mm	Glossy	Convex
S. cerevisiae	Light blue-grey; solid color	1-2 mm	Very glossy	Convex
T. candida	Medium blue-grey; darker in center	4-5 mm	Matte to glossy	Convex
Cryptococcus sp.	Light green or tan; solid color	1-2 mm	Matte	Convex

blue-green and blue-green colonies darkened. The *Cryptococcus* species only remained unchanged.

Tetrazolium chloride medium. The species *C. albicans* demonstrated the expected limited ability to reduce tetrazolium chloride; colonies were cream or salmon, very glossy, and measured 4-5 mm in diameter (Table 6). In mixed culture, *C. albicans* colonies were differentiable only from the *Cryptococcus* species. In the remaining mixed cultures, colony appearances were variably affected by the density of colony growth on the membrane surface. Extending the incubation time to 72 hours was of no aid in differentiation.

Nitro-blue tetrazolium medium. Whether in pure culture or in mixed culture, differentiation among the six yeast species on the nitro-blue tetrazolium medium was distinct. *Candida albicans* colonies were a unique bright medium blue, glossy, convex, and measured 2-4 mm in diameter. The other five yeast species ranged in color from lavender to dark purple (Table 7). Extending the incubation time to 72 hours resulted in no significant color changes.

Selectivity of Membrane Filtration Media

The bismuth-sulfite medium was slightly inhibitory to *C. albicans*; the membrane yielded a 95% recovery rate (Table 8). Unfortunately, the medium was not highly inhibitory to either *C. tropicalis* or *C. parapsilosis* which

Table 6. Differentiation among six yeast species in pure culture on the tetrazolium chloride medium, MF incubated at 25°C for 48 hours.

	Pigmentation	Diameter	Surface	Elevation
C. albicans	Cream to salmon; solid color	3-5 mm	Very glossy	Convex, spreading
C. tropicalis	Light to medium pink; solid color	2-4 mm	Glossy	Convex
C. parapsilosis	Medium to dark rose; solid color	2-4 mm	Glossy	Convex
S. cerevisiae	Light pink; solid color	1 mm	Glossy	Convex
T. candida	Light to medium rose; some darker in center	2-3 mm	Glossy	Convex
Cryptococcus sp.	Yellow; some pink centers	2-4 mm	Glossy	Convex

Table 7. Differentiation among six yeast species in pure culture on the nitro-blue tetrazolium medium, MF incubated at 25°C for 48 hours.

	Pigmentation	Diameter	Surface	Elevation
C. albicans	Bright medium blue; solid color	2-4 mm	Glossy	Convex
C. tropicalis	Dark violet; solid color	3-5 mm	Glossy	Convex
C. parapsilosis	Medium blue-lavender; solid color	1-2 mm	Glossy	Convex
S. cerevisiae	Dark purple; solid color	3-5 mm	Matte	Convex
T. candida	Lavender; solid color	1-2 mm	Glossy	Convex
Cryptococcus sp.	Light blue-lavender; solid color	2-3 mm	Matte	Convex

Table 8. Comparison of recovery among six yeast species in pure culture on experimental membrane filtration media with a control glucose-peptone medium; values represent average numbers of yeast cells recovered in triplicate determinations.

	C. albicans	*C. tropicalis*	*C. parapsilosis*	*S. cerevisiae*	*T. candida*	*Cryptococcus* sp.
Bismuth-sulfite	200	180	140	170	63	28
Control medium	210	250	160	180	71	120
Relative recovery	95%	72%	88%	94%	98%	23%
Phosphomolybdic acid	76	93	87	170	7	44
Control medium	89	92	120	200	93	52
Relative recovery	85%	100%	72%	85%	8%	85%
Tetrazolium chloride	110	33	89	37	130	46
Control medium	140	130	120	200	140	58
Relative recovery	78%	25%	74%	18%	93%	79%
Nitro-blue tetrazolium	140	130	110	100	90	67
Control medium	140	130	110	110	96	65
Relative recovery	100%	100%	100%	91%	94%	100%

cannot be easily distinguished from *C. albicans* in mixed cultures. The growth of the *Cryptococcus* species was severely restricted, 23% recovered, but this yeast is easily differentiated from *C. albicans* in mixed culture.

The recovery rate of *C. albicans* on the phosphomolybdic acid medium was 85% (Table 8). With the exceptions of *C. parapsilosis* and *T. candida* which yielded respective recovery rates of 72% and 8%, the recovery rates of the other yeast species is equal or superior to that of *C. albicans*.

The tetrazolium chloride medium was highly inhibitory to *C. albicans*, *C. tropicalis* and *S. cerevisiae* yielding respective recovery rates of 78, 25 and 18% (Table 8). This selection against *C. albicans* and difficulty in differentiating *C. albicans* in mixed culture combine to eliminate the practical application of this medium to quantify *C. albicans* populations from water.

The nitro-blue tetrazolium medium was not significantly selective against any of the six yeast species tested; only *S. cerevisiae* and *T. candida* demonstrated less than 100% recovery (Table 8).

A comparison of the percentage recovery rates for the six yeast species on the four experimental membrane filtration media is presented in Table 9.

Effect of antibiotics. The addition of 0.4 mg/ml cycloheximide and/or 0.05 mg/ml chloramphenicol to the

Table 9. Percentage recovery of six yeast species on experimental membrane filtration media relative to recovery on a glucose-peptone control medium.

	Bismuth-sulfite medium	Phospho-molybdic acid medium	Tetrazolium chloide medium	Nitro-blue tetrazolium medium
C. albicans	95	85	78	100
C. tropicalis	72	100	25	100
C. parapsilosis	88	72	74	100
S. cerevisiae	94	85	18	91
T. candida	89	8	93	94
Cryptococcus sp.	23	85	79	100
Average recovery rate, all yeasts	77%	72%	61%	98%

bismuth-sulfite, phosphomolybdic acid and nitro-blue tetrazolium medium inhibited the growth, within 48 hours, of all yeast species tested except *C. albicans*. The recovery of *C. albicans* was not significantly affected by the addition of antibiotics (Table 10).

Inhibition of background organisms. The nitro-blue tetrazolium medium demonstrated the highest degree of gross selectivity, 59 total colony forming units (CFUs), relative to the bismuth-sulfite and phosphomolybcid acid media, 90 and 159 total CFUs respectively (Table 11).

Bacterial growth on the nitro-blue tetrazolium medium was severely restricted; 0.6% and 1.2% of the CFUs were identified as bacteria after 48 and 72 hours incubation, respectively. The growth of filamentous fungi was detected only after 72 hours, represented 15.8% of total CFUs, and the small colony size did not interfere with the identification of *C. albicans*. One hundered percent of the seeded *C. albicans* cells were recovered and all five of the colonies selected for subculture were verified as *C. albicans* using carbohydrate assimilation patterns.

The apparent recovery rate of *C. albicans* on the bismuth-sulfite medium was 91%, however, two of the five colonies selected for subculture were identified as *C. rugosa*. No bacterial growth was demonstrated and the growth of filamentous fungi was minimal.

The phosphomolybdic acid medium yielded an apparent

Table 10. Comparison of recovery of *Candida albicans* on experimental membrane filtration media containing antibiotics with a control glucose-peptone medium; values represent average numbers of yeast cells recovered in triplicate determinations.

	C. albicans	*C. tropicalis*	*C. parapsilosis*	*S. cerevisiae*	*T. candida*	*Cryptococcus* sp.
Bismuth-sulfite with cyclo-heximide & chloramphenicol	91	0	0	0	0	0
Control medium	97	71	110	99	92	67
Relative recovery	94%	0%	0%	0%	0%	0%
Phosphomolybdic acid with cycloheximide	80	0	0	0	0	0
Control medium	97	71	110	99	92	67
Relative recovery	82%	0%	0%	0%	0%	0%
Nitro-blue tetrazolium with cycloheximide & chloramphenicol	96	0	0	0	0	0
Control medium	97	71	110	99	92	67
Relative recovery	99%	0%	0%	0%	0%	0%

Table 11. Comparison of recovery of *Candida albicans* from seeded surface water with recovery from seeded PBS on experimental membrane filtration media; values represent average numbers of yeast cells recovered in triplicate determinations.

	Bismuth-sulfite medium	Phospho-molybdic acid medium	Nitro-blue tetrazolium medium
C. albicans in PBS:	47	40	48
C. albicans in surface water:			
Total CFUs	90	159	59
C. albicans	43	35	48
Assessed recovery	91%	88%	100%

recovery rate of 88%; one of the five colonies selected for subculture was not *C. albicans* but an unidentified yeast. Filamentous fungal growth was severely restricted but bacterial growth was heavy.

Due to the excellent selectivity and sensitivity demonstrated by the nitro-blue tetrazolium medium in preliminary trials, this medium was used for the isolation and the enumeration of indigenous *C. albicans* from the selected surface water sources.

Quantification of *C. albicans*

Yeast density increased with linear flow downstream and with distance from the headgate in the irrigation ditch systems (Table 12). The occurrence of bacterial and filamentous fungal colonies was rare but occurrence did increase as water turbidity increased. Neither colony type posed a problem with differentiation of *C. albicans*; the bacterial colonies were mucoid and either colorless or yellow, the filamentous fungal colonies were small even after 72 hours incubation and were either purple or white.

Candida albicans was not detected in the Rattlesnake Creek but was consistently recovered from the irrigation ditch systems from mid-July through August when the coliform density peaks (122) (Table 13). The density of *C. albicans* in the Rattlesnake eastside and the major Missoula irrigation ditch systems did not exceed 7 and 15 *C. albicans*

Table 12. Total colony forming units recovered in the Rattlesnake Creek drainage and the Missoula irrigation ditch systems using the nitro-blue tetrazolium medium, MF.

	June 27	July 11	July 25	August 15
Woods Gulch	33	22	26	23
Lolo Street Bridge	52	48	38	42
Front Street Bridge	55	59	51	53
Rattlesnake School	96	92	128[b]	104[a]
Prescott School	94[a]	112[a]	154[a]	126[a]
Polk and Cherry	128[a]	82[a]	126[a]	122[a]
Orange Street	40	72[b]	65	54
Target Range School	178	208[a]	200[a]	234[a]
24th and Briggs	204[a]	240[a]	135[a]	276[a]
Floral Court	214[a]	238[a]	274[a]	302[a]

[a] Signifies positive identification of *C. albicans* using carbohydrate assimilation patterns following isolation.

[b] Signifies positive rapid demonstration of *C. albicans* using Mycosel enrichment broth from samples yielding negative results on *C. albicans* colony isolation on a membrane filter.

Table 13. Enumeration of *Candida albicans* in the Rattlesnake Creek drainage and the Missoula irrigation ditch systems; values represent the total number of *C. albicans* cells recovered in triplicate determinations of 500 ml volume each.

	July 25	August 1	August 8	August 15
Rattlesnake School	-	-	-	2
Prescott School	5	7	8	10
Polk and Cherry	6	7	9	9
Orange Street	-	1	-	-
Target Range School	2	-	6	7
24th and Briggs	7	11	16	19
Floral Court	13	16	19	23

cells per liter, respectively.

Of the 203 typical *C. albicans* colonies isolated, 199 colonies were confirmed as the organism and 4 were unidentified *Candida* species. Of the 30 atypical yeast colonies selected for identification, none were verified as *C. albicans* but were identified as *C. tropicalis*, *C. parapsilosis*, *C. pseudotropicalis*, *S. cerevisiae*, *C. rugosa*, and an unknown yeast. The colony color of the atypical colonies included dark to medium purple, blue, yellow and cream.

Assessed Cyst Recovery from the Membrane Filter Surface

The recovery of *S. cerevisiae* cells from the membrane filter surface ranged from 96 to 98% for the three procedure trials tested (Table 14). The number and the size of the yeast cell clumps present appeared to be the same in the original cell suspension and the recovered filtered suspensions. It was therefore concluded that, having vortexed the original cell suspension, the processes of filtration and resuspension did not have a significant effect on results through the dissolution of budding yeast cells.

Trial 1: The highest rate of cell recovery from the membrane, 98%, was achieved when the cells were resuspended in 1.0 ml phosphate buffer. Unfortunately, this procedure could not be successfully applied to the enumeration of *G. lamblia* cysts. When the concentrated suspended material

Table 14. Recovery of *Saccharomyces cerevisiae* cells from the membrane filter surface for three procedure trials; trial mean values determined using a hemacytometer are recorded as $n \times 10^5$ cells/ml.

	Trial Mean	Procedure Mean	Corrected Mean	Recovery
Original suspension:				
222,192,217,232,214	215			
217,220,172,206,198	203	207	-	-
191,209,188,213,248	210			
190,218,188,201,197	199			
Trial 1:				
101,120,121, 93,105	108			
94,117, 94, 94, 95	99	101	202	98%
90, 91,101,103, 95	96			
Trial 2:				
93, 74, 78, 83, 85	83			
77, 81, 79, 74, 74	77	79	198	96%
80, 74, 80, 73, 78	77			
Trial 3:				
128,142,130,128,132	132			
133,138,132,148,139	138	134	201	97%
107,145,132,141,135	132			

from the fecal specimen was resuspended in 1.0 ml phosphate buffer and examined microscopically using a hemacytometer, the debris present nagated cyst enumeration by obscuring or obliterating the morphological characteristics required for cyst identification.

Trial 2: The yeast cell recovery rate was 96% when the cells were resuspended in 5.0 ml phosphate buffer. Cyst enumeration was facilitated by decreasing the fecal debris concentration but due to the low number of cysts likely to be isolated from a natrual water source, this procedure becomes impractical: a decrease in debris concentration is concomitant with a decrease in the cyst concentration and a decrease in test sensitivity.

Trial 3: The yeast cell recovery rate remained high at 97% when the membrane was washed repeatedly and the cell suspensions were pooled. This high recovery rate indicated that no significant loss of cells occurred when the cell suspensions were transferred and pooled. The procedures of Trial 3 were easily applied to the enumeration of *G. lamblia* cysts.

Quantification of *G. lamblia* Cysts

Cyst recovery using membrane filtration and zinc sulfate concentration. The filtration of a fecal suspension aliquot followed by zinc sulfate concentration yielded a 91% recovery rate (Table 15). The slide preparations

Table 15. Enumeration of *Giardia lamblia* cysts in a fecal suspension using zinc sulfate concentration.

Procedure[a]	Cyst number per 0.5 ml	Mean	% Cysts recovered from membrane	% Cysts lost by prefiltration
Step 1	405,395,400	400	91[b]	
Step 2	366,358,367	364		47[d]
Step 3	211,208,217	212	92[c]	46[e]
Step 4	201,197,191	196		

[a] Refer to Figure 1 for procedure description.
[b] $Mean^2/Mean^1$
[c] $Mean^4/Mean^3$
[d] $(Mean^1 - Mean^3)/Mean^1$
[e] $(Mean^2 - Mean^4)/Mean^2$

contained very little debris and the cysts were easily identified microscopically by the comma-shaped parabasal bodies, deeply stained strands and the obvious presence of at least two nuclei.

Effect of prefiltration. The prefiltration of the fecal suspension to remove large debris resulted in a significant loss of the cysts present. Nearly 50% of the cysts were retained in the prefiltration membrane (Table 15). As prefiltration did not improve recovery, but rather resulted in a considerable loss, this procedure was abandoned. However, prefiltration did reveal that the debris present does not significantly hinder the recovery of cysts from the membrane filter surface nor does it interfere with zinc sulfate concentration. The recovery rates for the prefiltered and the non-prefiltered fecal suspensions were 92 and 91%, respectively (Table 15).

Cyst recovery from seeded surface water. The recovery of cysts from seeded surface water averaged 93.5% (Table 16). This high rate of recovery from turbid water was a further indication that the debris present did not significantly interfere with cyst recovery from the membrane surface or with zinc sulfate concentration.

Detection of indigenous cysts. The filtration of over 36,000 ml of water collected from Rattlesnake Creek, Lafray Creek, Rock Creek and Wallace Pond did not result in the successful isolation of *G. lamblia* cysts (Table 17). The

Table 16. Recovery of *Giardia lamblia* cysts from seeded surface water using membrane filtration and zinc sulfate concentration.

Original fecal suspension:	Aliquot number	Cyst count/ml	Mean
	1	548	
	2	643	601
	3	612	

Seeded surface water:	Membrane No.	Volume filtered	Cyst count	Recovery
Seed with 1.0 ml	1	300 ml[a*]		
	2	300 ml[a]	311[a]	
	3	200 ml[b]		92%
	4	200 ml[b]	240[b]	
Seed with 2.0 ml	1	300 ml[c]		
	2	300 ml[c]	697[c]	
	3	200 ml[d]		95%
	4	200 ml[d]	443[d]	

* Letter disignates those suspensions which were pooled prior to the zinc sulfate concentration procedure.

Table 17. Surface water collection sites and the total volume of water filtered at each site for the isolation of indigenous *Giardia lamblia* cysts.

Surface water source	Approximate volume filtered per membrane	Total volume filtered
Rattlesnake Creek	1,500 ml	4,500 ml
Lafray Creek	2,000 ml	9,500 ml
Rock Creek	2,000 ml	10,000 ml
Wallace Pond	1,000 ml	12,000 ml

turbidity of the water was no greater than that of the seeded surface water and was ruled out as a contributing factor in producing negative results.

The water sample collected at Wallace Pond did contain nematode eggs of the genera *Capillaria* (25 micrometers by 65 micrometers, polar plugs) and *Toxocara* (65 micrometers by 80 micrometers, thick, finely pitted shells) (119), which numbered in the hundreds. The nematode eggs are considerably larger than *G. lamblia* cysts (8 micrometers by 11 micrometers) but the zinc sulfate concentration procedures recommended for the isolation of nematode eggs and *Giardia* cysts are the same (14). It may therefore be concluded that had *G. lamblia* cysts been present in the collected water samples, they would have been detected with the isolation procedures used.

IV. DISCUSSION

Evaluation of the Experimental Membrane Filtration Media

Each of the four experimental membrane filtration media were evaluated in terms of selectivity and specificity for their practical application to the quantification of *C. albicans* from water.

Bismuth-sulfite medium. Selectivity on this medium was good, no bacterial growth was evident after 48 hours incubation at 36°C and filamentous fungal growth was minimal. The apparent recovery rate of *C. albicans* from seeded surface water, 91%, was significantly elevated due to a high frequency of false positive results; *C. albicans* could not be differentiated from *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis* or *C. rugosa* with sufficient accuracy to quantify the *C. albicans* cells present.

Phosphomolybdic acid medium. An 85% *C. albicans* recovery rate from cold stressed PBS was demonstrated on the phosphomolybdic acid medium. It has been demonstrated that the molybdate indicator is not inhibitory to the yeast (70,94), but that the nutritional characteristics of the medium confer its selective and differential properties (95). A substitution for source of carbon (glucose for

sucrose) and source of nitrogen (neopeptone for proteose peptone) increased the recovery of *C. albicans* but results were not satisfactory for differentiation. However, by increasing the proteose peptone concentration from the recommended 2% for membrane filtration media to 3%, the colonies were more uniform in size and color.

The differentiation among the six yeast species was good in pure and in bi-floral cultures but poor when filtering seeded surface water. The medium allowed for the heavy overgrowth of bacteria found in natural waters which affected the recovery rate and growth of the stressed yeast cells and their ability to reduce molybdate.

Tetrazolium chloride medium. Problems with this medium were demonstrated early with the poor differentiation among the six yeast species in bi-floral yeast cultures. The recovery rate of *C. albicans* from seeded cold stressed PBS on this medium was 78%, the lowest recovery rate demonstrated by the four experimental media.

Nitro-blue tetrazolium medium. This medium yielded an average *C. albicans* recovery rate of 100% from both seeded cold stressed PBS and seeded cold stressed surface water. The selective capability of the medium was excellent, reducing the bacteria and filamentous fungi present in surface water samples by at least 1,000 fold, and the differentiation of *C. albicans* was distinct.

Precision of the Yeast Recovery Method

The precision of the yeast recovery method was determined from D^2 values for assay variability (49) calculated according to the method of Eisenhart and Wilson (1943) as follows:

$$D^2 = \frac{N \Sigma x_i - (\Sigma x_i)^2}{\Sigma x_i} \quad \text{where,}$$

Σx_i is the summation of the SGA spread-plate or experimental membrane filtration media counts x_1, x_2 ... x_n and N (the number of replicate plates or filters per sample) was three. The D^2 values calculated for 21 spread-plated and 24 membrane filtered samples are shown in Figure 4, together with the expected D^2 control limits for P = 0.5, 0.2 and 0.05.

As can be seen, assay variability approximated that expected by chance alone. It would have been expected that 23, 9 and 2 of the D^2 values exceed the control limits of 1.4, 3.2 and 6.0; the actual number of D^2 values which did exceed the control limits were 16, 7 and 1.

Since the introduction of microbiological water quality assessment in the late 1800s, several criteria have been developed to define an indicator organism (Table 19) (11,40,97,106,134). When these criteria are considered, *C. albicans* has potential as an indicator organism when utilizing membrane filtration and the nitro-

Figure 4. Precision of yeast recovery procedures as estimated from the dispersion of D^2 values. (---; control limits when probability is as stated). Data from triplicate plates and filters were used to calculate D^2 values for each point (see Appendix).

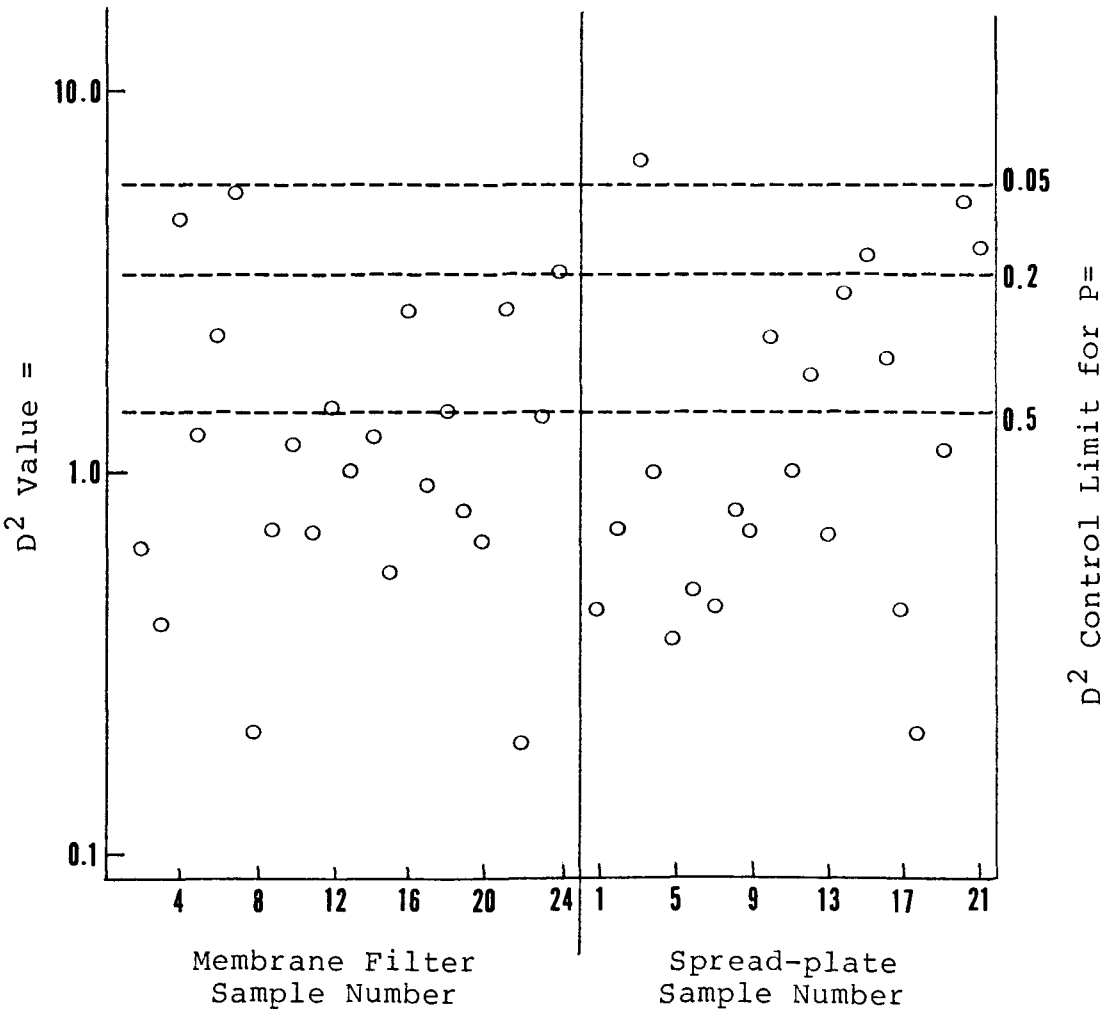


Table 18. Criteria defining an indicator organism.

Indicates fecal contamination.
More numerous than the pathogen.
More resistant than the pathogen to the aqueous environment.
Independent of other organisms in the aqueous environment.
Does not proliferate more than the pathogen in the aqueous environment.
At least 75% are recovered from seeded, cold stressed water.
Isolation procedures reduce background organisms by three orders of magnitude.
Isolation procedures demonstrate favorable statistical properties.

blue tetrazolium medium for the enumeration of *C. albicans* from water.

Quantification of *C. albicans* in Surface Water

The differentiation of *C. albicans* detected in the surface water samples was distinct; colonies were a bright medium blue, glossy and convex, bacterial growth was severely restricted and the growth of filamentous fungi was minimal.

As the total microbial population increased with distance from the headgate in the Rattlesnake eastside and the major Missoula irrigation ditch systems, the density of *C. albicans* cells also increased (Table 20). The number of *C. albicans* cells detected at the Prescott School and at Polk and Cherry, 4.5 to 5.0 km from the headgate, was sixteen times the number of *C. albicans* cells detected at Rattlesnake School, 0.5 km from the headgate. This increase in water contamination may be attributed to the grazing lands, manured gardens and private septic systems located between the Rattlesnake and Prescott schools.

The density of *C. albicans* at 24th and Briggs and at Floral Court is nearly four times the density at the Target Range School although all three sites are approximately 6.5 km from the headgate. The higher *C. albicans* density at the 24th and Briggs and Floral Court sites may be attributed to the animal stables and animal pastures

Table 19. Comparison of the average total colony forming units recovered and the average *Candida albicans* colony count for the dates July 25th and August 15th in relation to distance from the headgate.

Water sample collection site	Distance from the headgate	Number of colony forming units	Number of *C. albicans*
Rattlesnake School	0.5 km	116	1
Prescott School	4.5 km	140	8
Polk and Cherry	4.5 km	124	8
Target Range School	6.5 km	217	5
24th and Briggs	6.5 km	206	13
Floral Court	6.5 km	288	18

near these sites.

The specificity of the nitro-blue tetrazolium medium was confirmed by the infrequency of false-positive colonies (2%) and the absence of false-negative colonies.

Detection of *G. lamblia* Cysts

The membrane filtration and zinc sulfate concentration procedures presented in this report yielded a 96% recovery rate of *G. lamblia* cysts from seeded surface water indicating that membrane filtration and zinc sulfate concentration provide a feasible approach to the detection of cysts in water. It was also demonstrated that increased water turbidity of heavily contaminated water had no deletarious affect on cyst recovery whereas prefiltration to remove large debris resulted in nearly a 50% loss of the cysts present.

Correlation Between the Occurrence and Density of *C. albicans* and *G. lamblia*

Membrane filtration and zinc sulfate concentration have been used successfully to confirm the presence of cysts in a suspected water source during a waterborne giardiasis epidemic (41,69,113), but these procedures have not been used successfully to assess health risk from waterborne giardiasis.

A positive correlation between the occurrence and density of *C. albicans* and the degree of water contamina-

tion has been demonstrated and quantitative *C. albicans* data may be used to assess giardiasis risk. Unfortunately, no indigenous *G. lamblia* cysts were detected and no correlation between the occurrence and density of these two organisms in water could be formulated.

V. SUMMARY

A biological water quality assessment technique is judged in terms of sensitivity, specificity and resistence. The sensitivity of any assessment technique depends upon the number of indicator organisms which can be isolated from a sample of reasonable volume. The coliforms present in a fecal specimen outnumber the yeasts present by a factor of fifty (79,108), but the survival indices of these organisms in the aqueous environment and the significance of cell number recovered must be considered when using either organism as an indicator of water quality.

The recovery of *C. albicans* on the membrane filter is a more accurate representation of the actual aquatic population than is represented by the coliform number recovered on the membrane filter. A high total bacterial count is indicative of coliform suppression and as pollution increases the rapid overgrowth of non-fecal bacterial types nullifies the accurate identification of the coliforms present (10,52,67). In contrast, the nitro-blue tetrazolium medium demonstrated excellent selectivity and specificity when used to quantify *C. albicans* from water. The growth of bacteria and filamentous fungi was severely restricted, background organisms were reduced by at least

three orders of magnitude, and water turbidity did not affect the recovery of *C. albicans* which averaged 100% from seeded, cold stressed surface water samples. The frequency of false positive and false negative colonies was low at 2% and 0%, respectively.

The membrane filtration and zinc sulfate concentration procedures yielded a 96% recovery rate of *G. lamblia* cysts from seeded surface water indicating that the procedures developed were sound, but that dilution of contamination necessitates the use of large volume samples to increase test sensitivity.

The organisms *C. albicans* and *G. lamblia* both demonstrate long term survival outside the animal host, do not proliferate in the aqueous environment, are specific indications of contamination from human or animal sources, and act independently of the presence of other organisms in the aqueous environment. Therefore, quantitiative *C. albicans* data could be applied to the assessment of health risk from waterborne giardiasis.

Both organisms are also more resistant than coliforms to water disinfection procedures. The yeast and cysts remain viable after exposure to chlorine levels which are lethal to *Escherichia coli* (18,35,46,81,91). Coliform enumeration procedures are not applicable to chlorinated effluents and among the most common features shared by waterborne giardiasis outbreaks is the use of water where chlorination is the principle method of disinfection (80).

The use of a chlorine resistant indicator such as *C. albicans* would facilitate the assessment of giardiasis risk from chlorinated effluents as well as untreated surface waters.

The results of this report have demonstrated a positive correlation between the occurrence and density of *C. albicans* in water and the degree of water contamination. As attempts to isolate indigenous *G. lamblia* cysts were unseccussful, a correlation between the occurrence and density of *C. albicans* and *G. lamblia* in water requires further investigation.

APPENDIX

BiGGy Agar (Difco):

yeast extract	1.0 g
glycine	10.0 g
dextrose	40.0 g
bismuth-sulfite indicator	8.0 g
agar	20.0 g

Dissolve in 1 liter distilled water and heat to about 80°C to disperse the agar. The medium was agitated prior to pouring the plates to distribute the precipitate.

Phosphomolybdic Acid Plating Medium (MacLaren & Armen, 1958):

proteose peptone	10.0 g
sucrose	40.0 g
agar	15.0 g

Dissolve in a liter distilled water and after adjusting to pH 7.6 with 8% aqueous NaOH, autoclave for 15 min at 15 lbs pressure. To 100 ml of the basal medium, cooled to 50 to 55°C, add 1.5 ml of a 12.5% solution of filter-sterilized phosphomolybdic acid. The medium was agitated prior to pouring the plates to distribute the precipitate.

Tetrazolium Chloride Plating Medium:

neopeptone	10.0 g
yeast extract	1.0 g
glucose	40.0 g
agar	15.0 g

Dissolve in 1 liter distilled water and after adjusting to pH 6.0 with 8% aqueous NaOH, autoclave for 15 min at 15 lbs pressure. To 100 ml of the basal medium, cooled to 50 to 55°C, add 1.0 ml of a 2.5% solution of filter-sterilized 2,3,5-triphenyl-2H-tetrazolium chloride.

Nitro-Blue Tetrazolium Plating Medium:

phytone peptone	10.0 g
yeast extract	1.0 g
glucose	40.0 g
agar	15.0 g

Dissolve in 1 liter distilled water and after adjusting to pH 6.0 with 8% aqueous NaOH, autoclave for 15 min at 15 lbs pressure. To 100 ml of the basal medium, cooled to 50 to 55°C, add 1.0 ml of a 2.5% solution of filter-sterilized tetrazolium blue.

Sabouraud Glucose Agar (Difco):

polypeptone	10.0 g
dextrose	40.0 g
agar	15.0 g

Dissolve in 1 liter distilled water and autoclave for 15 min at 15 lbs pressure.

Mycosel Broth:

phytone peptone	10.0 g
dextrose	10.0 g
cycloheximide	0.4 g
chloramphenicol	0.05 g

Dissolve in 1 liter distilled water and autoclave for 15 min at 15 lbs pressure.

Glucose-Peptone Yeast Broth:

polypeptone	2.0 g
glucose	8.0 g
yeast extract	0.2 g

Dissove in 100 ml distilled water and after adjusting to pH 5.6 with 8% aqueous NaOH, autoclave for 15 min at 15 lbs pressure.

Phosphate Buffered Saline Solution:

potassium di-hydroxide phosphate ..... 34.0 g
sodium chloride ..... 10.0 g

Dissolve in 500 ml distilled water and adjust to pH 7.2 with 1 N aqueous NaOH. Dilute to 1000 ml with additional distilled water. Add 1.2 ml of stock phosphate buffer saline solution to 1000 ml distilled water and autoclave for 15 min at 15 lbs pressure.

Phosphate Buffer Solution:

potassium di-hydroxide phosphate ..... 34.0 g

Dissolve in 500 ml distilled water and adjust to pH 7.2 with 1 N aqueous NaOH. Dilute to 1000 ml with additional distilled water. Add 1.25 ml of stock phosphate buffer solution to 1000 ml distilled water and autoclave for 15 min at 15 lbs pressure.

Zinc Sulfate Solution:

zinc sulfate ..... 33.1 g

Dissolve in 100 ml distilled water and adjust to disired specific gravity using a hydrometer and adding additional distilled water or additional zinc sulfate.

Lugol's Iodine Solution:

iodine ..... 5.0 g
potassium iodide ..... 10.0 g

Dissolve the potassium iodide into 100 ml distilled water and add the iodine crystals slowly and shake to dissolve.

Data Used to Calculate D^2 Values:

	C. albicans	*C. tropicalis*	*C. parapsilosis*	*S. cerevisiae*	*T. candida*	*Cryptococcus* sp.
Bismuth-sulfite	202	173	138	155	58	23
Medium, MF	188	188	148	158	61	26
	207	180	140	191	70	34
	(199) *	(180)	(142)	(168)	(63)	(28)
D^2 value	.97	.63	.39	4.48	1.24	2.32
Phosphomolybdic	62	89	81	164	7	38
Acid Medium, MF	73	94	92	183	6	44
	91	95	87	168	9	50
	(75)	(93)	(87)	(172)	(7)	(44)
D^2 value	5.72	.22	.70	1.17	.70	1.64
Tetrazolium	103	32	83	31	137	48
Chloride Medium,	105	37	92	33	127	50
MF	117	29	91	44	122	39
	(108)	(33)	(89)	(36)	(129)	(46)
D^2 value	1.06	1.27	.55	2.72	.91	1.50
Nitro-Blue	134	140	99	97	82	57
Tetrazolium	147	129	115	103	91	63
Medium, MF	147	128	123	98	98	77
	(143)	(132)	(112)	(99)	(90)	(66)
D^2 value	.97	.67	2.67	.20	1.40	3.40

* Number in parentheses designates mean value.

Data Used to Calculate D^2 Values (cont):

	C. albicans	*C. tropicalis*	*C. parapsilosis*	*S. cerevisiae*	*T. candida*	*Cryptococcus sp.*
SGA Control for Bismuth-sulfite Medium, MF	207 204 217 (209)	256 259 241 (252)	178 134 169 (160)	187 181 168 (179)	75 68 70 (71)	116 126 125 (122)
D^2 value	.44	.74	6.67	1.06	.37	.50
SGA Control for Phosphomolybdic Acid Medium, MF	93 84 89 (89)	87 91 99 (92)	127 114 118 (120)	185 210 213 (203)	86 92 100 (93)	57 44 54 (52)
D^2 value	.46	.81	.74	2.33	1.06	1.79
SGA Control for Tetrazolium Chloride Medium, MF	136 148 133 (139)	146 124 121 (130)	96 119 123 (113)	98 111 119 (109)	146 136 137 (140)	58 60 55 (58)
D^2 value	.68	2.87	3.76	2.06	.44	.22
SGA Control for Nitro-blue Tetrazolium Medium, MF	130 148 139 (140)	-	-	-	97 80 112 (96)	56 62 78 (65)
D^2 value	1.18				5.34	3.98

LITERATURE CITED

1. Ahearn, D.G. 1974. Identification and ecology of yeasts of medical importance, pp. 129-146. In: J.E. Prier and H. Friedman (eds.), Opportunistic Pathogens, University Park Press, Baltimore.

2. Ahearn, D.G. 1978. Medically important yeasts. Ann. Rev. Microbiol. *32*:59-68.

3. Ahearn, D.G., F.J. Roth and S.A. Meyers. 1968. Ecology and characterization of yeasts from aquatic regions of south Florida. Marine Biology. *1*:291-308.

4. Ajello, L. 1956. Soil as a natural reservoir of human pathogenic fungi. Science. *123*:876-879.

5. Anderson, J.H. 1979. *In vitro* survival of human pathogenic fungi in seawater. Sabouraudia. *17*:1-12.

6. Bailey, W.R., and E.G. Scott. 1974. Laboratory diagnosis of mycotic infections, pp. 237-275. In. Diagnostic Microbiology, 4th ed., C.V. Mosby Co, St. Louis.

7. Barbour, A.G., C.R. Nichols and T. Fukushima. 1976. An outbreak of giardiasis in a group of campers. Amer. J. Tropical Med. Hyg. *25*:384-389.

8. Beech, F.W., and J.G. Carr. 1955. A survey of inhibitory compounds for the separation of yeasts and bateria in apple juices and ciders. J. Gen. Microbiol. *12*:85-94.

9. Bissonnette, G.K., J.J. Jekeski, G.A. McFeters and D.G. Stuart. 1977. Recovery characteristics of bacteria injured in the natural aquatic environment, pp. 98-100. In: R.H. Bordner, C.F. Frith and J.A. Winter (eds.), Proceedings of the symposium on recovery of indicator organisms employing membrane filters, U.S. Environmental Protection Agency, EPA 600/9-77-024, EMSL-Cincinnatti, Cincinnatti, Ohio.

10. Black, R.E., M.A. Horwitz and G.F. Craun. 1978. Outbreaks of waterborne disease in the United States, 1975. J. Infect. Dis. *137*:370-374.

11. Bonde, G.J. 1966. Bacteiological methods for estimation of water pollution. Health Lab Sci. *3*:124-128.

12. Borchardt, J.A., and G. Walton. 1971. Water quality, pp. 1-51. In: Water quality and treatment handbook: a handbook of public water supplies, American Water Works Association, 3rd ed., McGraw-Hill Co, New York.

13. Bordner, R., J. Winter and R. Scarpino, Eds. 1978. Microbial methods for monitoring the environment: water and wastes. U.S. Environmental Protection Agency, EPA 600/8-78-017, EMSL-Cincinnatti, Cincinnatti, Ohio.

14. Braswell, J.R., and A.W. Hoadley. 1974. Recovery of *Escherichia coli* from chlorinated secondary sewage. Appl. Microbiol. *28*:328-329.

15. Brown, H.W. 1979. Clinical parasitology, 4th ed., Appleton-Century-Crofts, New York.

16. Buck, J.D. 1975. Distribution of aquatic yeasts - effects of incubation temperature and chloramphenicol concentration on isolation. Mycopath. *56*:73-79.

17. Buck, J.D. 1976. Pollution microbiology of Biscayne Bay beaches. Florida Sci. *39*:111-120.

18. Buck, J.D. 1977. *Candida albicans*, pp. 139-147. In: A.W. Hoadley and B.J. Dutka (eds.), Bacterial indicators/health hazards associated with water, ASTM STP 635, Amer. Soc. Testing and Materials, Philadelphia.

19. Buck, J.D. 1980. Occurrence of human-associated yeasts in the feces and pool waters of captive bottlenosed dolphins (*Tursiops truncatus*). J. Wildlife Dis. *16*:141-149.

20. Buck, J.D., and P.M. Bubucis. 1978. Membrane filter procedure for enumeration of *Candida albicans* in natural waters. Appl. Environ. Microbiol. *35*:237-342.

21. Buckley, H.R. 1971. Fungi pathogenic for man and animals: the subcutaneous and deep seated mycoses, pp. 461-478. In: C. Booth (ed.), Methods in Microbiology, vol 4, Academic Press, New York.

22. Buckley, H.R., C.K. Campbell and J.C. Thompson. 1969. Techniques for the isolation of pathogenic fungi, pp. 113-126. In: D.A. Shapton and G.W. Gould (eds.), Isolation Methods for Microbiologists, Academic Press, New York.

23. Bump, C.M., and L.J. Kunz. 1968. Routine identification of yeasts with the aid of molybdate agar medium. Appl. Microbiol. *16*:1503-1506.

24. Carney, J.F., C.E. Carty and R.R. Colwell. 1975. Seasonal occurrence and distribution of microbial indicators and pathogens on the Rhode River of Chesapeake Bay. Appl. Microbiol. *30*:771-780.

25. Chang, S.L., and P.W. Kabler. 1956. Detection of cysts of *Entamoeba histolytica* in tap water by the use of membrane filters. Amer. J. Hyg. *64*:170-180.

26. Clark, H.F., E.E. Geldreich, H.L. Jeter and P.W. Kabler. 1951. The membrane filter in sanitary bacteriology. Public Health Reports. *66*:951-977.

27. Cohen, R., F.J. Roth, E. Delgado, D.G. Ahearn and M.H. Kalser. 1969. Funfal flora of the normal human small and large intestine. New England J. Med. *280*:638-641.

28. Combs, T.J., R.A. Murchelano and F. Jurgen. 1971. Yeasts isolated from Long Island Sound. Mycologia. *63*:178-181.

29. Cooke, W.B. 1970. Our mouldy earth. A study in the fungi of our environment with emphasis on water. Robert A. Taft Research Center, Dept. of the Interior, Cincinnatti, Ohio.

30. Cooke, W.L. 1980. Selective isolation of *C. albicans* from fresh waters, p. 178. In: Abstracts of the Annual Meeting, American Society for Microbiology, 80th Meeting, New York.

31. Costa, S.O.P., and C. deLourdes Branco. 1964. Evaluation of a molybdenum culture medium as selective and differential for yeasts. J. Pathol. Bacteriol. *87*:428-431.

32. Craun, G.F. 1977. Waterborne outbreaks. J. Water Pollution Control Fed. *49*:1268-1278.

33. Craun, G.F. 1978. Waterborne outbreaks of giardiasis, pp. 127-147. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

34. Craun, G.F. 1979. Waterborne disease outbreaks in the United States. J. Environ. Health. *41*:259-265.

35. Craun, G.F., and L.J. McCabe 1973. Review of the causes of waterborne disease outbreaks. J. Water Works Asso. *65*:74-84.

36. Craun, G.F., L.J. McCabe and J.M. Hughes. 1976. Waterborne disease outbreaks in the United States, 1971-1974. J. Amer. Water Works Asso. *68*:420-424.

37. Crow, S.A., D.G. Ahearn and W.L. Cooke. 1975. Densities of bacteria and fungi in costal surface films as determined by a membrane-adsorption procedure. Limnol. Oceanog. *20*:644-645.

38. Dancigar, M., and M. Lopez. 1975. Numbers of *Giardia* in the feces of infected children. Amer. J. Tropical Med. Hyg. *24*:237-242.

39. Davies, R.B., and C.P. Hibler. 1978. Animal reservoirs and cross-species transmission of giardiasis, pp. 104-125. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

40. diMenna, M.E. 1955. A search for pathogenic species of yeasts in New Zealand soils. J. Gen. Microbiol. *12*:54-62.

41. Dutka, B.J. 1973. Coliforms are an inadequate index of water quality. J. Environm. Health. *36*:39-43.

42. Dykes, A.C., D.D. Juranek, R.A. Lorenz, S. Sinclair, W. Jakubowski and R. Davies. 1980. Municipal waterborne giardiasis: an epidemiological investigation. Annals Internal Med. *92*:165-170.

43. Dzawachiszwili, N., J.W. Landau, V.D. Newcomer and O.A. Plunkett. 1964. The effect of sea water and sodium chloride on the growth of fungi pathogenic to man. J. Investigative Dermatol. *43*:103-109.

44. Eisenhart, C., and P.W. Wilson. 1943. Statistical methods and control in bacteriology. Bacteriol. Rev. *7*:57-137.

45. Ekundayo, C.A. 1980. Effects of sodium chloride and seawater in Sabouraud dextrose agar medium on some human pathogenic fungi. Canadian J. Bot. *57*:2418-2121.

46. Eliassen, R., and R.H. Cummings. 1948. Analysis of waterborne outbreaks, 1938-1945. J. Amer. Water Works Asso. *40*:509-515.

47. Engelbrecht, R.S., D.H. Foster, E.O. Greening and S.H. Lee. 1974. New microbial indicators of wastewater chlorination efficiency. U.S. Environmental Protection Agency, EPA 670/2-73-082, NERC-Cincinnatti, Cincinnatti, Ohio.

48. Fell, J.W., and S.A. Meyer. 1967. Systematics of yeast species in the *Candida parapsilosis* group. Mycopathol. Mycol. Appl. *32*:177-193.

49. Fell, J.W., and N. vanUden. 1963. Yeasts in marine environments, pp. 329-340. In: C.H. Oppenheimer (ed.), Symposium of marine microbiology, C.C. Thomas, Springfield.

50. Fisher, R.A. 1950. Statistical methods for research workers, 11th ed., Hafner Publishing Co, New York.

51. Gallagher, T.P., and D.F. Spino. 1968. The significance of numbers of coliform bacteria as an indicator of enteric pathogens. Water Research. *2*:169-175.

52. Geldreich, E.E. 1970. Applying bacteriological parameters to recreational water quality. J. Amer. Water Works Asso. *62*:113-120.

53. Geldreich, E.E. 1972. Waterborne pathogens, pp. 207-241. In: R. Mitchell (ed.), Water pollution microbiology, Wiley-Interscience, New York.

54. Geldreich, E.E. 1977. Performance variability of membrane filter procedures, pp. 12-19. In: R.H. Bordner, C.F. Frith and J.A. Winter (eds.), Proceedings of the symposium on recovery of indicator organisms employing membrane filters. U.S. Environmental Protection Agency, EPA 600/9-77-024, EMSL-Cincinnatti, Cincinnatti, Ohio.

55. Geldreich, E.E., H.L. Jeter and J.A. Winter. 1967. Technical considerations in applying the membrane filter procedure. Health Laboratory Sci. *4*:113-125.

56. Geldreich, E.E., H.D. Nash, D.J. Reasoner and R.H. Taylor. 1972. The necessity of controling bacterial populations in potable waters: community water supply. J. Amer. Water Works Asso. *64*:596-602.

57. Georg, L.K., L. Ajello and C. Papageorge. 1954. Use of cychloheximide in the selective isolation of fungi pathogenic to man. J. Laboratory and Clinical Med. *44*:422-428.

58. Goetz, A., and N. Tsuneishi. 1951. Application of molecular filter membranes to the bacteriological analysis of water. J. Amer. Water Works Asso. *43*:943-984.

59. Gordon, M.A., and H.B. Cupp. 1953. The detection of *Histoplasma capsulatum* and other fungus spores in the environment by means of the membrane filter. Mycologia. *45*:241-252.

60. Gorman, A.E., and A. Wolman. 1939. Waterborne outbreaks in the United States and their significance. J. Amer. Water Works Asso. *31*:225-228.

61. Grabow, W.O.K., J.S. Burger and E.M. Nupen. 1980. Evaluation of acid-fast bacteria, *Candida albicans*, enteric viruses and conventional indicators for monitoring wastewater reclamation systems. Water Sci. Tech. *13*:803-817.

62. Grant, D.R., and T.K. Woo. 1978. Comparative studies of *Giardia* spp. in small mammals in Southern Ontario. II. Host specificity and infectivity of stored cysts. Canadian J. Zool. *56*:1360-1366.

63. Green, R.A., R.H. Bordner and P.V. Scarpino. 1974. Applicability of the membrane filter and most probable number coliform procedures to chlorinated wastewaters, p. 34. In: Abstracts of the Annual Meeting, American Society for Microbiology, 74th Meeting, New York.

64. Greenberg, A.E., and H.J. Ongerth. 1966. Salmonellosis in Riverside, California. J. Amer. Water Works Asso. *58*:1145-1150.

65. Guarraia, L.J. 1973. Fungi: a state of the art, pp. 106-111. In: S.S. Verner (ed.), Proceedings of the first microbiology seminar of standardization of methods. U.S. Environmental Protection Agency, Washington, D.C.

66. Hawkes, P., V.J. Tipton, C.C. Hofheins, C.R. Nichols, M.A. Smith and T. Fukushima. 1974. Giardiasis-Utah. Morb. Mort. Wkl. Rept. *23*:397-398.

67. Hayes, A., and R. Thompson. 1963. Identification of *Candida albicans*. Amer. J. Clinical Pathol. *40*:553-555.

68. Hayes, S.P., and D.G. Ahearn. 1963. Water pollution in the Yellowstone River drainage in Montana. Progress Report #63, Montana State Board of Health, Helena, Montana.

69. Hendricks, C.W., and S.M. Morrison. 1967. Multiplication and growth of selected enteric bacteria in clear mountain stream water. Water Research. *1*:567-576.

70. Hibler, C.P., K. McLeod and D.O. Lyman. 1975. Giardiasis in residents of Rome, New York, and in U.S. travelers to the Soviet Union. Morb. Mort. Wkl. Rept. *24*:366-371.

71. Holland, M.L., and L.J. Kunz. 1964. Evaluation of molybdate agar as a selective, differential medium for yeasts. J. Bacteriol. *81*:869-874.

72. Hugham, J.B. 1974. Evaluating the membrane fecal coliform test by using *Escherichia coli* as the indicator organism. Appl. Microbiol. *27*:771-776.

73. Intestinal Parasite Surveillance - U.S. 1976. 1978. Bureau of Epidemiology, Parasitic Diseases Division, Atlanta, GA. Morb. Mort. Wkl. Rept. *27*:167-168.

74. Iralu, V. 1974. Laboratory diagnosis of intestinal parasites, pp. 279-309. In: W.R. Bailey and E.G. Scott (eds.), Diagnostic microbiology, 4th ed., C.V. Mosley Co, St. Louis.

75. Jakubowski, W., S. Chang, T.H. Ericksen, E.C. Lippy and E.W. Akin. 1978. Large-volume sampling of water supplies for microorganisms. J. Amer. Water Works Asso. *70*:702-706.

76. Jakubowski, W., and T.H. Ericksen. 1978. Methods for detection of *Giardia* cysts in water supplies, pp. 104-125. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

77. Jarroll, E.L., A.K. Bingham and E.A. Meyer. 1980. *Giardia* cyst destruction: effectiveness of six small quantity water disinfection methods. Amer. J. Tropical Med. Hyg. *29*:8-11.

78. Jarroll, E.L., A.K. Bingham and E.A. Meyer. 1981. Effect of chlorination on *Giardia lamblia* cyst viability. Appl. Environm. Microbiol. *41*:483-487.

79. Juranek, D. 1978. Waterborne giardiasis, pp. 150-161. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

80. Kapica , L. , and A. Clifford. 1966. Quantitative determination of yeasts in sputum. Mycopathol. Mycol. Appl. *34*:27-32.

81. Keifer, A., G. Lynch, R.R. Roberts, C. Powers, J, Goston, R.W. Sherwood, L. Johnson, J. Blair, B. Early, R.S. Hopkins, E. Scott, R. Smade, H. Osterud, N. Slifman, J. Schilke, C. Hills, J.A. Googins, T.J. Berkhart, J. Jenkins, E.W. Witte, M. McCarthy, T. deMelfi and E.C. Lippy. 1980. Waterborne giardiasis - California, Colorado, Oregon, Pennsylvania. Morb. Mort. Wkl. Rept. *29*:121-123.

82. Kirner, J.C., J.D. Littler and L.A. Angelo. 1978. A waterborne outbreak of giardiasis in Camas, Washington. J. Amer. Water Works Asso. *70*:35-40.

83. Kishimoto, R.A., and G.E. Baker. 1969. Pathogenic and potentially pathogenic fungi isolated from beach sands and selected soils of Oahu, Hawaii. Mycologia. *61*:539-548.

84. Klein, D.A., and S. Wu. 1974. Stress: a factor to be considered in hetertrophic microorganism enumeration from aquatic environments. Appl. Microbiol. *27*:429-431.

85. Kott, Y. 1977. Current concepts of indicator bacteria, pp. 3-13. In: A.W. Hoadley and B.J. Dutka (eds.), Bacterial indicators/health hazards associated with water, ASTM STP 635, Amer. Soc. Testing and Materials, Philadelphia.

86. Leclerc, H., D.A. Mossel, P.A. Trinel and F. Gavini. 1977. Microbiological monitoring - a new test for fecal contamination, pp. 23-34. In: A.W. Hoadley and B.J. Dutka (eds.), ASTM STP 635, Amer. Soc. Testing and Materials, Philadelphia.

87. Levine, N.D. 1978. *Giardia lamblia*: classification, structure, identification, pp. 2-8. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

88. Lin, S. 1973. Evaluation of coliform tests for chlorinated secondary effluents. J. Water Pollution Control Fed. *45*:498-506.

89. Lin, S. 1977. Comparison of membranes for fecal coliform recovery in chlorinated effluents. J. Water Pollution Control Fed. *49*:2255-2264.

90. Lippy, E.C. 1978. Tracing a giardiasis outbreak at Berlin, New Hampshire. J. Amer. Water Works Asso. *70*:512-520.

91. Liu, P., and A. Newton. 1955. Rapid chlamydospore formation by *Candida albicans* in a buffered alkaline medium. Amer. J. Clin. Pathol. *25*:93-97.

92. Lopez, C.E., A.C. Dykes, D.D. Juranek, S.P. Sinclair, J.M. Conn, R.W. Christie, E.C. Lippy, M.G. Schultz and M.H. Mires. 1980. Waterborne giardiasis: a community wide outbreak of disease and a high rate of asymptomatic infection. Amer. J. Epidemiol. *112*:495-507.

93. Lopez, C.E., D.D. Juranek, S.P. Sinclair and M.G. Schultz. 1978. Giardiasis in American travelers to Madera Island, Portugal. Amer. J. Tropical Med. Hyg. *27*:1128-1132.

94. Mackinzie, D.W. 1971. Effect of relative humidity on survival of *Candida albicans* and other yeasts. Appl. Microbiol. *22*:678-682.

95. MacLaren, J.A. 1960. The cultivation of pathogenic fungi on a molybdenum medium. Mycologia. *52*:148-152.

96. MacLaren, J.A., and D. Armen. 1958. Pigmentation of *Candida albicans* by molybdenum. Amer. J. Clinical Pathol. *30*:411-422.

97. Matson, E.A., S.G. Horner and J.D. Buck. 1979. Effect of upgrading a municipal wastewater effluent on pollution indicator and other microorganisms in river water. Environm. Sci. Technology. *13*:460-465.

98. McCabe, L.J. 1977. Epidemiological consideration in the application of indicator bacteria in North America, pp. 18-27. In: A.W. Hoadley and B.J. Dutka (eds.), Bacterial indicators/health hazards associated with water, ASTM STP 635, Amer. Soc. Testing and Materials, Philadelphia.

99. McFeters, G.A., and D.G. Stuart. 1972. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. Appl. Microbiol. *24*:805-811.

100. Meyer, E.A., and E.L. Jarroll. 1980. Giardiasis. J. Epidemiol. *111*:1-12.

101. Meyer, S.A., and F.J. Roth. 1961. Isolation and survival of *Candida* species in a marine environ, p. 71. In: Abstracts of the Annual Meeting, American Society for Microbiology, 61st Meeting, New York.

102. Meyers, S.P., D.G. Ahearn and W.L. Cooke. 1970. Mycological studies of Lake Champlain. Mycologia. *62*:504-515.

103. Moore, G.T., W.M. Cross, D. McGuire, G.R. Healy and L.H. Newton. 1969. Epidemic giardiasis at a ski resort. New England J. Med. *281*:402-407.

104. Nickerson, W.J. 1953. Reduction of inorganic substances by yeast. J. Infect. Dis. *93*:43-56.

105. Nickerson, W.J., and Z. Mankowski. 1953. A polysaccharide medium of known composition favoring chlamydospore formation in *Candida albicans*. J. Infect. Dis. *92*:20-25.

106. Noel, J.G., and R.E. Simard. 1973. Yeast as an indicator of fecal and organic pollution, p. 33. In: Abstracts of the Annual Meeting, American Society of Microbiology, 73rd Meeting, New York.

107. Ott, W.R. 1978. Water quality indices: a survey of indices used in the United States. U.S. Environmental Protection Agency, EPA 600/4-78-005, Office of Research and Development, Washington, D.C.

108. Pegano, J.F., J.D. Levin and W. Trejos. 1957. Diagnostic medium for differentiation of species of *Candida*. Antibiotics Annual. *1957-1958*:137-143.

109. Prescott, S.C., C.A. Winslow and M.H. McCrady. 1946. Water bacteriology, 6th ed., John Wiley and Sons, Inc., New York.

110. Qureshi, A.A., and B.J. Dutka. 1976. Comparison of various brands of membrane filters for their ability to recover ungi from water. Appl. Environm. Microbiol. *32*:445-447.

111. Rendtorff, R.C. 1978. The experimental transmission of *Giardia lamblia* among volunteer subjects, pp. 171-180. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

112. Ross, E.C., K.W. Campbell and H.J. Ongerth. 1966. *Salmonella typhimurium* contamination of Riverside, California water supply. J. Amer. Water Works Asso. *58*:165-174.

113. Roth, F.J., D.G. Ahearn, J.W. Meyers and S.A. Meyer. 1962. Ecology and taxonomy of yeasts isolated from various maring substrates. Limnol. Oceanogr. *7*:178-185.

114. Shaw, P.K., R.E. Brodsky, D.C. Lyman, B.T. Wood, C.P. Hibler, G.R. Healey, K.I.E. MacLeod, W. Stahl and M.G. Schultz. 1977. A community wide outbreak of giardiasis with evidence of transmission by a municipal water supply. Ann. Intern. Med. *87*:426-432.

115. Shuval, H.I., J. Cohen and R. Kolodney. 1973. Regrowth of coliforms and fecal coliforms in chlorinated wastewater effluents. Water Research. *7*: 537-546.

116. Simard, R.E., and A.C. Blackwood. 1970. Yeasts from the St. Lawrence River. Canadian J. Microbiol. *17*:197-203.

117. Skinner, C.E., and D.W. Fletcher. 1960. A review of the genus *Candida*. Bacteriol. Rev. *24*:397-416.

118. Smith, R.J., and R.M. Twedt. 1971. Natural relationships of indicator and pathogenic bacteria in stream waters. J. Water Pollution Control Fed. *43*:2200-2209.

119. Smith, R.J., R.M. Twedt and L.K. Flanigan. 1973. Relationships of indicator and pathogenic bacteria in stream water. J. Water Pollution Control Fed. *45*:1736-1745.

120. Soulsby, E.J.L. 1968. Helminths, arthropods and protozoa of domesticated animals, 6th ed., pp. 593-596. Williams and Wilkins, Co, Baltimore.

121. Standridge, J.H. 1976. Comparison of surface pore morphology of two brands of membrane filters. Appl. Environm. Microbiol. *31*:316-319.

122. Stuart, D.G., G.A. McFeters and J.E. Schillinger. 1977. Membrane filter technique for the quantification of stressed fecal coliforms in the aquatic environment. Appl. Environm. Microbiol. *34*:42-46.

123. Taylor, J.J., and M.A. Neher. 1978. Missoula valley water study: report on sanitary bacteriological quality of selected surface and ground waters. University of Montana, Missoula, MT.

124. Taylor, J.J., and B.C. Tangen. 1968. Growth and antibiotic sensitivity of *Candida albicans* and enteric bacteria alone and in bifloral cultures. Montana Academy of Science Proc. *28*:46-50.

125. Taysi, I., and N. vanUden. 1964. Occurrence and population densities of yeast species in an estuarine-marine area. Limnol. Oceanog. *9*:42-45.

126. Tobin, R.S., and B.J. Dutka. 1977. Comparison of the surface structure, metal binding, and fecal coliform recoveries of nine membrane filters. Appl. Environm. Microbiol. *34*:69-79.

127. vanDonsel, D.J., E.E. Geldreich and N.A. Clarke. 1967. Seasonal variations in survival of indicator bacteria in soil and their contribution to stormwater pollution. Appl. Microbiol. *15*:1362-1370.

128. vanUden, N. 1960. The occurrence of *Candida* and other yeasts in the gastrointestinal tracts of animals. Annals New York Academy Sci. *89*:59-68.

129. vanUden, N., and D.G. Ahearn. 1963. Occurrence and population densities of yeast species in a fresh water lake. Antonie van Leeuwenhoek J. Microbiol. Serol. *29*:308-312.

130. vanUden, N., and R. Castelo Branco. 1963. Distribution and population of yeast species in pacific water, air, animals and kelp off Southern California. Limnol. Oceanog. *8*:323-329.

131. vanUden, N., M. deMatos Faia and L. Assis-Lopez. 1956. Isolation of *Candida albicans* from vegetable sources. J. Gen. Microbiol. *15*:151-153.

132. vanUden, N., L. doCarmo Sousa and M. Farinka. 1958. On the intestinal yeast flora of horses, swine, goats and sheep. J. Gen. Microbiol. *19*:435-445.

133. Veazie, L., B. Brownlee and H.J. Sears. 1978. An outbreak of gastroenteritis associated with *Giardia lamblia*, pp. 171-180. In: W. Jakubowski and J.C. Hoff (eds.), Waterborne transmission of giardiasis. Proceedings of a symposium, September 18-20, 1978. U.S. Environmental Protection Agency, EPA 600/9-79-001, Environmental Research Center, Cincinnatti, Ohio.

134. Walker, J.D., B.F. Conrad, P.A. Seesman and R.R. Colwell. 1977. Comparison of membrane filter counts and plate counts on heterotrophic and oil agar used to estimate populations of yeast, fungi and bacteria, pp. 178-187. In: R.H. Bordner, C.F. Frith and J.A. Winter (eds.), Proceedings of the symposium on recovery of indicator organisms employing membrane filters. U.S. Environmental Protection Agency, EPA 600/9-77-024, EMSL-Cincinnatti, Cincinnatti, Ohio.

135. Watkins, D.W., C.D. Thomas and V.J. Cabelli. 1976. Membrane filtration procedure for enumeration of *Vibrio parahaemolyticus*. Appl. Environm. Microbiol. *32*:679-684.

136. Wolf, H.W. 1972. The coliform count as a measure of water quality, pp. 333-345. In: R. Mitchell (ed.), Water pollution microbiology, Wiley-Interscience, Inc., New York.

137. Wolfe, M.S. 1975. Giardiasis. Amer. Med. Asso. J. *233*:1362-1365.

138. Wolfe, M.S. 1978. Giardiasis. New England J. Med. *298*:319-321.